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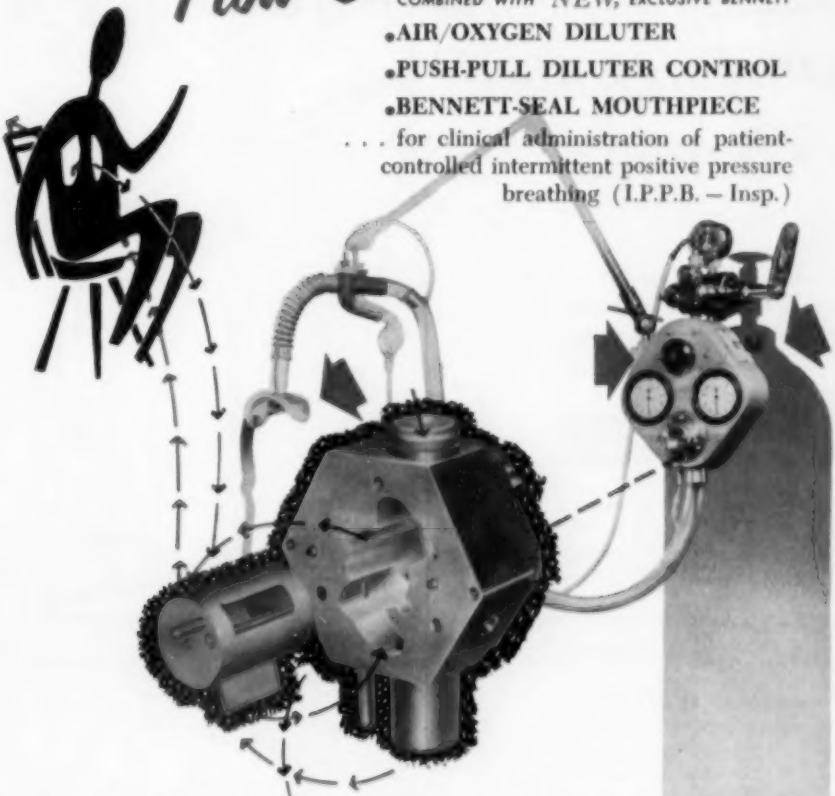

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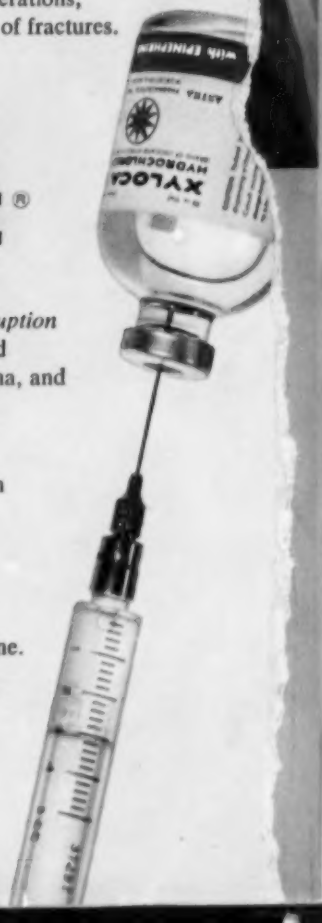

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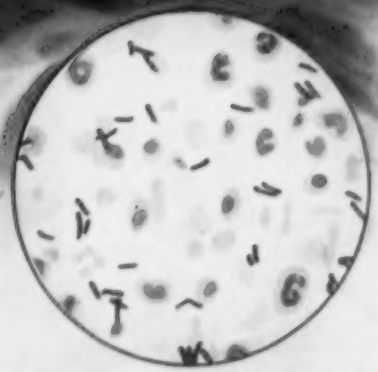

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VOLUME 73 *May, 1956* NUMBER 5

CHRONIC PROGRESSIVE (CAVITARY) HISTOPLASMOSIS AS A PROBLEM IN TUBERCULOSIS SANATORIUMS [1, 2, 3]

MICHAEL L. FURCOLOW[4] AND CHARLES A. BRASHER

(Received for publication November 2, 1955)

INTRODUCTION

Chronic progressive histoplasmosis, in contrast to the acute progressive or disseminated form, occurs primarily among older persons and is localized to the lungs except in the terminal stages. Both forms of the disease, however, are eventually fatal. The early stage of the chronic form is characterized by apical or subapical pneumonic infiltrations which progress to multiple thick-walled, usually bilateral cavities. This type of disease was first described by Johnson and Batson (1) and by Bunnell and Furcolow (2) in 1948. Subsequent experience with the original and later cases has confirmed the progressive nature of the disease (3). Sutliff and associates (4) reviewed seven cases from the literature and described four additional cases in 1953, but classified the disease as "active chronic" and benign. Six additional cases have been reported (3, 5–7).

In order to avoid further confusion in terminology the descriptive term, chronic progressive (cavitary) histoplasmosis, is proposed to designate this type of disease.

The symptoms of this form of histoplasmosis are cough, moderate weight loss, usually a slight elevation of the erythrocyte sedimentation rate, occasional temperature, profuse sputum, sometimes hemoptysis. The disease is characterized by exacerbations; the patient usually seeks medical help after an acute bout of what is called "influenza," at which time pneumonic or cavitary disease is found. This bout may subside and the patient not be seen again until several years later, at which time he has a similar episode and further extension is discovered. Quite often the cavitation is only in one lung at the start and, later

[1] From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, and the Missouri State Sanatorium, Mount Vernon, Missouri.

[2] Presented at the Fourteenth Veterans Administration-Army-Navy Conference on the Chemotherapy of Tuberculosis, Atlanta, Georgia, February 9, 1955.

[3] Presented, in part, before the Medical Session, as part of *Section 6B*, at the annual meeting of the National Tuberculosis Association, Milwaukee, Wisconsin, May 25, 1955.

[4] Chief, Kansas City Field Station, Communicable Disease Center, U. S. Public Health Service, University of Kansas Medical Center, Kansas City, Kansas.

on, following another bout of so-called "influenza," the disease spreads to the other lung. In the early stages large cystlike cavities may be seen, followed later by spread of the process.

The disease usually progresses to fatal termination and just before this occurs there is dissemination of the fungus throughout the body with enlargement of the liver and spleen. Frequently at post-mortem examination *Histoplasma* may be cultured from all organs of the body.

In this type of disease the sputum is usually loaded with *Histoplasma capsulatum* and positive cultures are readily obtained. Serologic tests are usually positive and serve as a good screening tool. Tubercle bacilli are occasionally found, but most of the cases are not complicated by tuberculosis.

Chronic progressive histoplasmosis closely resembles reinfection type of tuberculosis, and could be considered to be reinfection histoplasmosis. This classification is based on the finding in some cases of negative tuberculin tests, positive histoplasmin skin tests, positive serologic tests, positive cultures for the fungus, and the presence of chronic cavitary disease of the apices in association with calcified lesions in the hilar area. Autopsy on such patients fails to reveal evidence of tuberculosis and the criteria of "chronic reinfection histoplasmosis" appear to be fulfilled.

A total of only 16 cases of chronic progressive cavitary histoplasmosis has been previously reported. It is the purpose of the present paper to show that this type of disease occurs frequently among patients in a tuberculosis sanatorium.

MATERIAL AND METHODS

The studies to be reported have been conducted at the Missouri State Sanatorium at Mount Vernon, Missouri. This sanatorium has an average daily census of 550 patients, 85 per cent of whom are white and 15 per cent are Negro. Casual studies begun in 1952 revealed an occasional case of histoplasmosis. Between March and August, 1954, a systematic survey of the patients at the sanatorium was undertaken. During the survey, skin tests with tuberculin and histoplasmin were performed on all patients and serologic tests were made. The mycologic laboratory services were augmented and careful attempts were made to establish the diagnosis in all patients whose serologic tests were positive or whose diagnosis was in doubt in any way.

The tuberculin skin tests were performed with purified protein derivative (PPD-S), furnished by Dr. Florence Seibert of the Henry Phipps Institute, Philadelphia, Pennsylvania. A dose of 0.0001 mg. in 0.1 ml. was used. The histoplasmin used was 0.1 ml. of Lot HKC-5, diluted 2 parts to 1,000. This dose is equivalent to other standard histoplasmins. Readings of the skin tests were made at forty-eight hours, and any reaction showing an induration of 5 mm. or more was considered positive. The skin tests were administered and read by the ward physician in each case.

Serologic tests were performed by Dr. Samuel Salvin at Rocky Mountain Laboratory, U. S. Public Health Service, Hamilton, Montana, and by Dr. Joseph Schubert at the Communicable Disease Center Serological Laboratory, U. S. Public Health Service, Chamblee, Georgia. Dr. Salvin performed a complement-fixation test with yeast-phase *Histoplasma* organisms as antigen and a precipitin test with a purified histoplasmin antigen. Dr. Schubert performed a complement-fixation test with histoplasmin as antigen. A serologic test

was interpreted as positive if definite results were obtained in a serum dilution of 1:8 or higher.

Mycologic examinations were performed in the laboratory of the Missouri State Sanatorium and in the Kansas City Field Station. Specimens were shipped from Mt. Vernon to Kansas City in screw-capped vials with the addition of saline containing 10,000 units each of penicillin and streptomycin. Both cultures and mouse inoculation techniques were employed in the attempt to isolate the organism. Examinations for tuberculosis were performed in the laboratory in the Missouri State Sanatorium. Smears were usually employed although cultures were frequently done. Guinea pig injection was employed when the organisms were difficult to find or were atypical on culture.

Pathologic examinations were made by Dr. David Gorelick and Dr. Fred Coller of Springfield, Missouri.

In this paper a case was termed "mycologically proved" if organisms typical of *H. capsulatum* were isolated by culture from the patient or were seen in tissues removed surgically or at autopsy. A "serologically proved" case was one in which the serologic tests for histoplasmosis were positive (1:8 or higher) on either of the complement-fixation tests or the precipitin test. Adequate cultural studies on the "serologically proved" cases usually revealed the presence of the organism. However, in many of these cases the patient had left the hospital before mycologic studies could be completed, or the disease was of the milder type from which positive cultures could not be obtained because of passage of time since infection.

RESULTS

These essentially casual studies for the presence of histoplasmosis in a tuberculosis hospital have now yielded a total of 19 "mycologically proved" cases and 95 "serologically proved" cases. These cases have been observed over a three-year period. During the three-month survey, 14 "mycologically proved" cases and 29 "serologically proved" cases were found. Of the 600 patients in the survey, 14 (2.4 per cent) were proved to have histoplasmosis by actual demonstration of the organism. In an additional 29 (4.8 per cent), the diagnosis of histoplasmosis was established by serologic tests.

In table 1 are shown the detailed findings in the group of 19 "mycologically proved" cases. It may be noted that in most cases the patients were more than fifty years of age, that the males outnumbered the females, and that all but one of the patients were white. In a number of cases, the onset of illness had occurred a number of years prior to determination of the etiology. The chest roentgenographic findings in almost all cases showed bilateral cavitation. Skin tests were positive to tuberculin in 9 cases and to histoplasmin in 14 of the 18 tested. The serologic tests for histoplasmosis were positive in every patient. The organism was identified from every patient in most cases by culture from the sputum; occasionally, from cultures from the lung at operation or autopsy. The presence of the organism was demonstrated in tissue obtained at post-mortem examination in 2 cases in which fungal cultures were not done.

The patients have been observed from a minimum of several months to a maximum of seventy-six months. Three patients have died. It will be noted that progression of the disease occurred in 9 of the 19 cases. In the last column are indicated the numbers of microscopic and cultural examinations for tubercle bacilli which were made on each patient.

TABLE 1

PROVED CASES OF HISTOPLASMOSIS—STATE SANATORIUM

Name	Age	Sex	Race	Onset Date	First Roentgenogram	Chest Roentgenographic Findings	Skin Tests		C. F. Serology	H. Culture	Length of Observation, Months	Course	Lesions on Last Roentgenogram	Tubercle Bacilli	
							Tuberculin	Histoplasmin						Smear	Culture
J.G.	62	M	W	11/49	12/50	Multiple cavities	+	+	+	+ S*	58	Alive	Progressive	10	3
S.D.	67	F	W	5/51	8/52	Bilateral cavities	−	+	+	+ S	7	Died, April, 1953	No change	13	5
S.R.	58	F	W	1/52	1/53	Multiple cavities R.L.	−	+	+	+ S	32	Neumonectomy, R. alive	Improved	19	2
A.E.	56	M	W	12/48	12/48	Bilateral cavities	+	+	+	+ S + L†	66	Operated; died August, 1954	Progressive	24	7
W.D.	60	M	W	10/53	12/53	Cavity R.U.L.	−	+	+	+ S	21	Alive	Progressive	34	16
F.S.	32	M	W	10/53	10/53	Bilateral cavities	+	+	+	+ S	24	Alive	No change	19	6
H.L.	60	M	W	11/49	1/50	Bilateral infiltration with cavity R	−	+	+	+ S	69	Alive	Progressive	44	52
H.F.	43	M	W	8/53	11/53	Cavity R.U.L.	−	+	+	+ L + S	7	Lobectomy February, 1954	Progressive	22	7
J.McC.	61	M	W	2/51	1/54	Multiple cavities	+	−	+	+ S	55	Alive	Progressive	16	16
H.J.	45	M	W	11/52	5/53	Bilateral cavities	−	+	+	+ S	28	Alive	Progressive	36	8
H.G.	48	M	W	1951	11/53	Cavity R	−	+	+	+ S	30	Alive	Progressive	21	3
G.M.	46	M	W	11/53	12/53	Bilateral infiltration with cavity R	+	−	+	Pathology, + L	6	Operated; died May, 1954	No change	24	7
E.M.	52	F	N	6/52	2/53	Bilateral infiltration with cavity R	+	ND	ND	Pathology, + L	24	Operated, October, 1953	Improved	6+ 40−	4+ 12−
H.H.	37	M	W	9/53	9/53	Infiltration? Cavity L		+	+	+ L	24	Operated, April, 1954	Improving	25	5
D.B.	43	M	W	10/53	12/53	Bilateral cavities	+	+	+	+ L	23	Operated, June, 1954	No change	13	4
G.M.	62	M	W	1944	11/48	Bilateral cavities	+	+	+	+ S	76	Alive	Progressive	40	5
V.M.	44	M	W	8/54	10/54	Bilateral infiltration with cavity R	+	+	+	+ S	11	Alive	No change	18	4
U.H.	58	M	W	2/55	2/55	Bilateral infiltration with cavity R	−	−	+	+ S	7	Alive	No change	7	2
L.F.	71	M	W	12/54	1/55	Bilateral infiltration with cavity L	−	−	+	+ S	8	Alive	No change	10	4

*S = Culture from sputum †L = Culture from lung

Detailed case descriptions and therapy trials will be discussed in separate papers. Hence, these subjects will not be discussed in detail in the present report. However, the reproductions of chest roentgenograms and brief clinical histories of 6 patients are shown in figures 1 to 9 at the end of this report. These serve to illustrate the type of lesion seen and its chronic character. The cavitary nature of the infection, the tendency to progress to bilateral involvement, and the usual presence of calcified lesions may all be seen from examination of the cases presented in the figures.

DISCUSSION

The finding that 7.2 per cent of the patients in a tuberculosis sanatorium have "serologically proved" histoplasmosis and that the actual presence of the microorganism was demonstrated in 33 per cent of the cases is indeed remarkable. It certainly calls for very serious consideration of the importance of histoplasmosis in tuberculosis sanatoriums. If it be assumed that a similar prevalence obtained throughout the general area of high histoplasmin sensitivity, quite startling figures would be obtained.

Calculations have been made of the number of tuberculosis sanatorium beds in the areas where histoplasmin sensitivity among adults exceeds 50 per cent. This area extends roughly from southern Ohio in a sweeping circle through central Indiana and Illinois, eastern Kansas and Oklahoma, northeast Texas, northern Louisiana, Mississippi and Alabama, and includes all of Tennessee, Arkansas, Missouri, and Kentucky. In this area it has been estimated that approximately 21 million people live. This region has approximately 16,500 sanatorium beds for tuberculosis. If one applies the figures arrived at in the Missouri State Sanatorium, it is estimated that approximately 400 "mycologically proved" cases of histoplasmosis might be found in these sanatoriums.

In addition, 800 cases of "serologically proved" histoplasmosis might be found. In short, if the findings of the present study prove to be generally applicable to the histoplasmosis area, some 1,200 patients in tuberculosis hospitals would be found to have histoplasmosis. These 1,200 cases would obviously furnish an important reservoir for differential diagnosis as well as a complicating factor in any therapeutic trial of tuberculosis.

The accurate estimation of this problem is obviously of fundamental importance to all persons concerned with the care of the tuberculous. These patients not only occupy tuberculosis beds; they derive no benefit from antituberculosis therapy and, indeed, may contract the disease. Furthermore, the demonstration of more cases of histoplasmosis should furnish an adequate pool of cases for trials of therapeutic agents so urgently needed in this fungal infection. Moreover, the finding of histoplasmosis in the degree which might be encountered in this area might furnish an answer to the decreasing prevalence of tuberculosis and permit further usage to be found for beds in these sanatoriums. At present the general treatment of histoplasmosis is similar to that of tuberculosis before specific therapy was found.

That these suggestions are not too far out of line has already been confirmed by Sutliff and Campbell (8) who recently reported the finding of 7.3 per cent

positive serologic tests for histoplasmosis among 150 tuberculous patients in Memphis, Tennessee.

SUMMARY

The prevalence of histoplasmosis of the chronic progressive (cavitary) type in a tuberculosis sanatorium is presented. A clinical description of the disease is given and diagnostic suggestions are made.

Studies of the prevalence of both "mycologically proved" cases and "serologically proved" cases are presented. The frequency of "mycologically proved" cavitary cases was 2.4 per cent among the 600 patients in the hospital, and the frequency of the "serologically proved" cavitary cases was 4.8 per cent. The implications of these findings may be seen by applying them to the 16,500 tuberculosis beds in the area of high histoplasmin sensitivity. If similar findings prevailed in the other sanatoriums, 1,200 cases of chronic progressive cavitary histoplasmosis might be expected to be found in these sanatoriums.

Suggestions are made for determining the prevalence of this disease among this group of patients. If the above predictions prove true, an adequate reservoir of patients for therapeutic trials would be available.

SUMARIO

La Histoplasmosis Evolutiva Crónica (Cavitaria) como Problema en los Sanatorios para Tuberculosos

Describese la frecuencia de la histoplasmosis de forma evolutiva crónica (cavitaria) en un sanatorio para tuberculosos. Se ofrece una descripción clínica de la dolencia y se presentan varias sugestiones para el diagnóstico.

Preséntanse estudios de la frecuencia de los casos tanto "micológicamente comprobados" como "serológicamente comprobados." La incidencia de casos cavitarios comprobados "micológicamente" fué de 2.4 por ciento entre los 600 enfermos del hospital y la de los comprobados "serológicamente" de 4.8 por ciento. Puede verse el alcance de estos hallazgos aplicándolos a los 16,500 lechos para tuberculosos en la zona de alta sensibilidad a la histoplasmina. Si fueren semejantes los hallazgos en los demás sanatorios, cabría esperar que hubiera en ellos 1,200 casos de histoplasmosis cavitaria evolutiva crónica.

Ofrecense sugestiones para determinar la frecuencia de esta dolencia en este grupo de enfermos. Si se cumplen las predicciones anteriores, habría a mano una reserva adecuada de enfermos para ensayos terapéuticos.

RESUME

Histoplasmose chronique évolutive en qualité de problème des sanatoriums de la tuberculose

La fréquence de l'histoplasmose sous la forme chronique évolutive (cavitaire), dans un sanatorium de la tuberculose, est exposée. Les auteurs fournissent une description clinique de l'affection et des suggestions quant au diagnostic.

Les études sur la fréquence des cas "mycologiquement" confirmés et de ceux "sérologiquement" confirmés sont présentées. La fréquence des cas cavitaires "mycologiquement" confirmés était de 2,4 % pour les 600 malades de l'hôpital et la fréquence des cas cavitaires "sérologiquement" confirmés s'élevait à 4,8 %. Les implications de ces résultats apparaissent lorsqu'ils sont appliqués aux 16.500 lits de la tuberculose dans les sanatoriums d'une région où l'allergie à l'histoplasmine est élevée. Si des résultats similaires étaient obtenus dans les autres sanatoriums, on pourrait s'attendre à découvrir 1.200 cas d'histoplasmose dans ces sanatoriums.

Les auteurs indiquent les moyens qui permettraient de déterminer la fréquence de cette maladie parmi ce groupe de malades. Dans le cas où ces présomptions recevraient confirmation, on disposerait d'un réservoir adéquat de malades pour les essais thérapeutiques.

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Fig. 1

Fig. 2

Figs. 1 and 2. W. M., age 61, was admitted to the hospital in February, 1951, complaining of loss of weight, tiredness, and chest pain of fifteen months' duration. The roentgenogram (figure 1) revealed bilateral pulmonary infiltrations with calcifications in the left hilus. Ten sputum examinations were negative for acid-fact bacilli, and three cultures were negative. Tuberculin and histoplasmin skin tests were positive; coccidioidin and blastomycin tests were negative. Bronchial smears were negative for tubercle bacilli. Serologic tests for histoplasmosis were negative. Cultures for *H. capsulatum* were positive. The patient left the hospital against advice after two months. He refused to return, but recently he returned to his physician with a complaint that he was feeling ill and losing weight and appetite. A roentgenogram obtained in 1954 (figure 2) reveals considerable extension of the disease on the left with apparently some contraction on the right. There still appear to be cavities in the right lung by planigram. Recent serologic tests for histoplasmosis were doubtfully positive.

FIG. 3. W. F., age 58, was admitted to the sanatorium in February, 1953, with complaints of fatigue, loss of weight, and increasing productive cough following an attack of "viral" pneumonia thirteen months previously. She had considerable sputum, occasionally streaked with blood and often purulent. In January her symptoms increased, with nausea, vomiting, and pain in the left upper quadrant; she was referred to the sanatorium. A chest roentgenogram revealed pulmonary cavitation and infiltration in the right upper lobe. Calcification in the hilar areas was also evident. There is evidence of an old pleurisy at the right base. Nineteen sputum specimens were negative for acid-fast bacilli by microscopy and two by culture. Bronchial smear was also negative for tubercle bacilli. Skin tests were positive for histoplasmin, and negative for tuberculin, blastomycin, and coccidioidin. Following increase in the pulmonary disease, a right pneumonectomy was performed on September 25, 1953. Cultures of the excised lung were positive for *H. capsulatum*. Sputum was positive on several occasions for *H. capsulatum*. Complement-fixation tests for histoplasmosis were also positive. Following surgery the patient had a gradual weight increase of 15 pounds. Roentgenograms of the lungs have shown no evidence of spread of the disease. *H. capsulatum* was seen in the pathologic specimens. The patient has shown steady improvement since operation. Serologic tests for histoplasmosis continue positive.

(When the initials "W. M." and "W. F." appear in the legends of the figures, they indicate "white male" and "white female," respectively.)

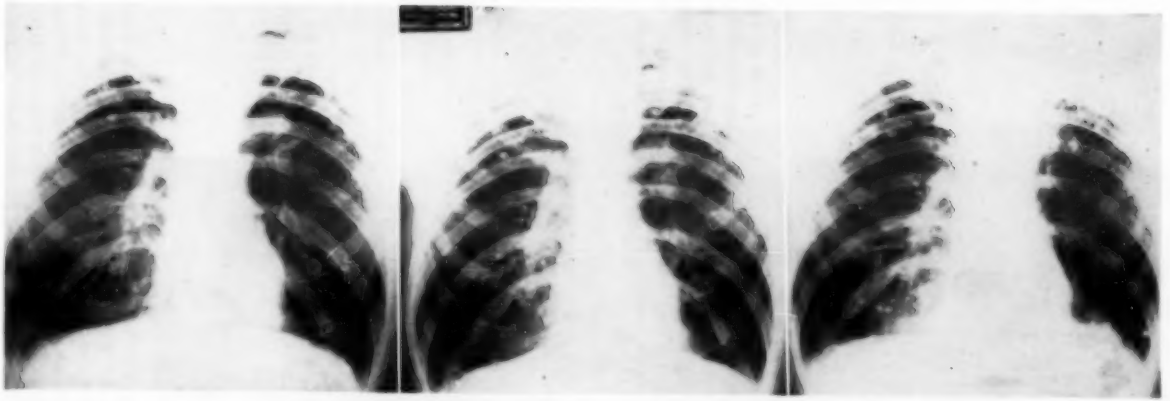

FIG. 4 FIG. 5 FIG. 6

FIGS. 4, 5, and 6. W. M., age 56, was first seen in December, 1948, and was referred to the sanatorium because routine fluoroscopic study revealed pulmonary disease. A roentgenogram obtained at this time showed bilateral apical infiltration with calcification in several areas throughout the lung fields (figure 4). The patient had had progressive dyspnea since 1945. In 1952 he became considerably worse following the cutting of silage in an open field; had chills and fever, weakness, and weight loss with productive cough. Histoplasmin and tuberculin skin tests were positive; blastomycin and coccidioidin were negative. A total of 24 smears was negative for acid-fast bacilli and 7 cultures were negative for tubercle bacilli. Complement-fixation tests for histoplasmosis were strongly positive and, in December, *H. capsulatum* was isolated from the sputum. Four sputum cultures on this patient were positive for *H. capsulatum*. A chest roentgenogram (figure 5) revealed extension of his disease on the left. Cavitation was later demonstrated and the disease progressed in some areas and contracted in others (figure 6). On August 13, 1954, a pneumonolysis of the left lung was done preparatory to lobectomy. However, this was not performed due to the extensive disease found. The patient was returned to the ward in poor condition and died three days later in postoperative shock. Autopsy revealed chronic granulomatous pneumonitis of the lung due to histoplasmosis and postoperative hemorrhagic enterocolitis. No evidence of tuberculosis was found at autopsy.

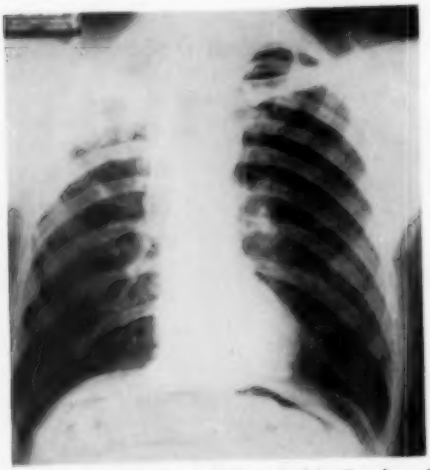

FIG. 7. W. M., age 59, was admitted to the hospital in October, 1953, because of chills and fever of approximately ten days' duration. The patient had lost about 30 pounds. The tuberculin skin test was negative; histoplasmin was positive. Complement-fixation tests for histoplasmosis were positive. Roentgenograms revealed marked infiltration in the right upper lobe with cavitation. Cultures were positive for *H. capsulatum* in December, 1953, and in May, 1954, on at least three different occasions. Smears and cultures for tubercle bacilli were negative. Twenty-one months of observation have shown no significant change in pulmonary findings.

FIG. 8. W. M., age 31, whose illness began with chills, fever, productive cough, and weakness in October, 1953, had lost 22 pounds. A chest roentgenogram revealed pulmonary infiltration and he was sent to the tuberculosis sanatorium. Nineteen sputum specimens for acid-fast bacilli between November, 1953, and January, 1954, were negative. Six sputum cultures were negative for tubercle bacilli. Tuberculin and histoplasmin skin tests were positive. Blastomycin and coccidioidin were negative. Two gastric specimens were negative for *M. tuberculosis* on culture. Sputum was positive for *H. capsulatum* on a number of occasions; first on January 11, 1954. Complement-fixation test for histoplasmosis was positive in January, 1954, and subsequently. Roentgenographic examination shows bilateral pulmonary apical infiltration with bilateral cavitation. Observation for two years has not shown significant change.

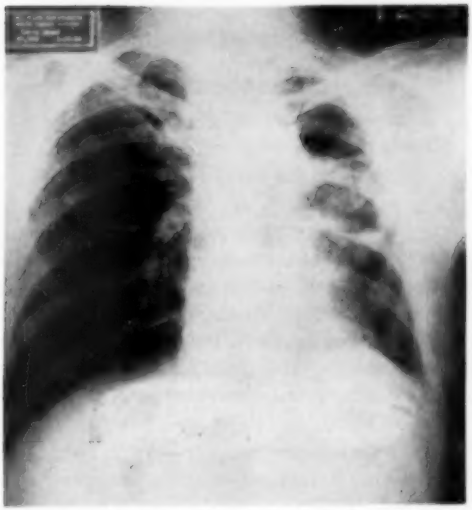

FIG. 9. W. M., age 60, was admitted to the hospital in December, 1953, with a three-year history of weakness, easy fatigue, cough, and sputum. In December, 1953, he had a massive hemoptysis and was admitted to the sanatorium. A roentgenogram revealed infiltration in the first interspace on the right, and the entire upper part of the left lung was occupied by cavities. There was a large cystlike cavity occupying the apex. Skin test with histoplasmin was positive; with tuberculin, negative. Cultures were positive for *Histoplasmin* on at least two occasions. Serologic tests for histoplasmosis were positive. The patient's first admission to a hospital was in January, 1950. A roentgenogram at that time showed infiltration and cavitation which has shown some progression in the last several years. He has been observed for a total of sixty-nine months. A total of 12 sputum specimens has been negative for acid-fast bacilli on microscopy, and two cultures have been negative for tubercle bacilli.

SENSITIVITY TO FUNGAL ANTIGENS AMONG STUDENTS AT THE UNIVERSITY OF MINNESOTA[1,2]

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INTRODUCTION

In 1927, one of the present writers reported that, among 4,500 children under observation who did not react to tuberculin, some presented roentgenographic evidence of calcium deposits in the chest (1). The specificity of the tuberculin test had already been so thoroughly demonstrated that this apparent discrepancy was thought to be due to such factors as "overreading" roentgenograms and, in nontuberculous disease, the deposition of calcium. Through the later work of such writers as Frimann-Dahl and Waaler (2), Aronson and associates (3), Palmer (4), Christie and Peterson (5), and Carter (6), it is now well recognized that deposition of calcium is not specific for any one condition or disease. Thus, it is impossible to determine the etiology of calcific deposits by the shadows they cast on roentgenograms or by gross inspection at necropsy or biopsy.

When Palmer (4) and Christie and Peterson (5) in 1945 and 1946 demonstrated that a large number of apparently healthy persons with evidence of calcium deposits in the chest reacted to histoplasmin, they suspected that, at least in the areas where they worked, histoplasmosis in a mild form was a common occurrence. After Palmer's demonstration, the present writers began testing with histoplasmin university students and others who had unexplained pulmonary calcifications.

REVIEW OF THE LITERATURE

The first report of a case of histoplasmosis in the world's literature was by Darling (7) in 1906. Diagnosis was made at necropsy of a young man from Martinique. Twenty years later Watson and Riley (8) described the first case in the United States. The patient was a resident of Hennepin County, in which Minneapolis is located. Between 1926 and 1945 less than 100 cases were reported in the world's literature. Therefore, the disease was regarded as rare and uniformly fatal. This belief was also maintained as it pertained to Minnesota even after Palmer (4) did the initial testing on life-long Minnesota students of nursing and found that only 4.1 per cent reacted. However, in 1945, Iams and associates (9) reported the death of an infant from Saint Paul. This was the second case reported in Minnesota.

In 1948, Rutledge (10) observed six cases in one farm family in Becker County, Minnesota. All had pulmonary lesions and all survived. The same year an elderly person died

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[2] Supported, in part, by a contract from the Veterans Administration upon recommendation of the National Research Council's Committee on Veterans Medical Problems; also supported, in part, by the H. Longstreet Taylor Foundation established by the Minnesota Tuberculosis and Health Association.

from histoplasmosis in Hennepin County. In 1949 a case was reported in St. Louis County, one from Dakota, one from Redwood, one from St. Louis, and one from Wabash. None of the patients died. In 1952 one case was reported from Hennepin and one from St. Louis County; both patients survived. Two cases were reported in 1953: one from Hennepin County and one from Renville County; both patients lived. A third case from Hennepin County was that of an elderly person who died.

In August, 1953, Smith (11) reported an acute fatal case in a four-year-old farm boy residing in Wright County. A two and one-half-year-old sibling had had an unexplained pulmonary condition during the past three months. It was then learned that six adult members of this family had had simultaneous pulmonary disease of varying degrees of severity in February, 1949. All 1949 data were assembled, and re-examinations in 1953, including roentgenographic inspections of the chest and histoplasmin tests, left little doubt that each of these persons had had histoplasmosis in 1949.

Smith (12) reported on histoplasmin and tuberculin testing surveys in Wright County. The first was in the vicinity of the outbreak which occurred in 1949. Testing 100 volunteers in October, 1953, revealed that, among 76 who had contact with the farm where the outbreak had occurred, 26.3 per cent reacted to histoplasmin. Later (May, 1954) these tests were offered to all of the children in public and parochial schools of the county. Eighty-five per cent were tested (5,487 children), of whom 7.5 per cent reacted to histoplasmin and 1.2 per cent to tuberculin. Among 199 adults, including teachers and other school personnel, 19.1 per cent reacted to histoplasmin and 25 per cent to tuberculin.

The epidemics described by Rutledge and Smith in Minnesota apparently were similar to the one described in northwestern Indiana by Loosli and associates (13) who were the first to describe such an epidemic which had been studied during the illness of the patients.

More recently other cases of clinical histoplasmosis have been diagnosed in Minnesota which have not yet appeared in the literature.

There appears little doubt that, in the past, clinical cases of histoplasmosis were treated in sanatoriums just as sarcoidosis and pulmonary malignancy were before refinements were available in differential diagnosis. This belief is strengthened by observations of Furcolow and Brasher (14) who found 2.4 per cent of active histoplasmosis and 4.8 per cent of probable cases among 600 persons treated for tuberculosis in a sanatorium.

The present survey revealed a higher incidence of histoplasmin reactors over the greater part of Minnesota than had been suspected, with unusually high percentages in the above-mentioned counties (figure 1). At first this was thought to be due to the fact that so many students residing in these counties had spent time in areas where histoplasmosis was then known to be endemic. However, subsequent findings have revealed that there is more histoplasmosis in the state than had previously been realized.

Consequently, when in 1947–1948 the opportunity arose to do a survey for fungal diseases among the Minnesota students along with a tuberculosis survey, it was welcomed. Dr. Carroll E. Palmer of the United States Public Health Service suggested that tests with fungal antigens be administered in addition to the tuberculin tests in a contemplated tuberculosis survey among the students. Although the fungal antigen test had not previously been used, Dr. Palmer pre-

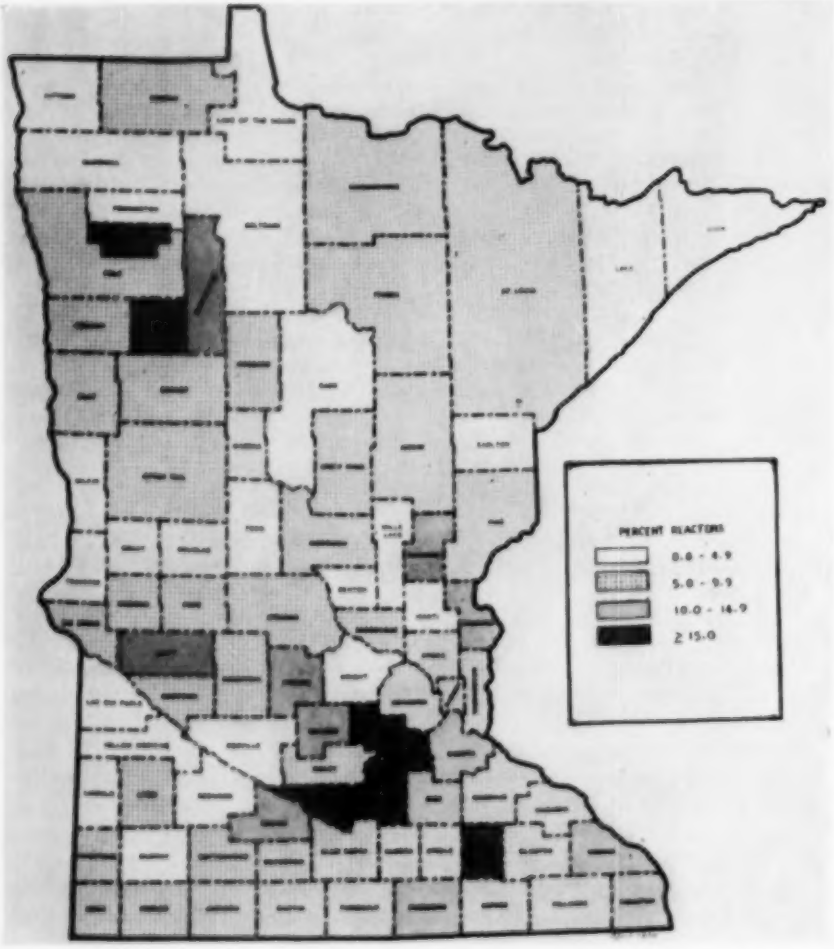

FIG. 1. Map of Minnesota counties showing histoplasmin reactor rates (data of table 7).

pared a triple antigen (blastomycin, coccidioidin, and histoplasmin) to be administered cutaneously in the same manner as tuberculin. Those students who reacted to triple antigen were to be tested with blastomycin, coccidioidin, and histoplasmin separately.

PLAN OF INVESTIGATION

The subjects surveyed consisted of 13,386 male students at the University of Minnesota, of whom less than 99 per cent were white. The majority were World War II veterans. As approximately 16,600 such students were enrolled at the University during the period of testing, the writers succeeded in seeing more than 80 per cent of those eligible for study.

The testing program was not compulsory for all students and, while repeated attempts were made to obtain the cooperation of those who failed to report following the first request, 19.5 per cent of the eligible men could not be tested. While some measure of self-selection on medical grounds may have entered into individual failures to cooperate, it is the belief of the writers that these were not of sufficient magnitude to bias their results. It was found, for example, that few of the students knew the meaning of "fungal antigen tests." Certainly few had previously been tested with these antigens, so that for the great majority there could have been no knowledge of reactor or nonreactor status which might have influenced the decision to cooperate or not.

The information elicited from each student included, in addition to such routine information as age, race, marital status, college, and class, a rather complete residence history which specified the four longest residences by county (not including the period of military service, if any) and characterized each period by duration and character as "farm dwelling," "non-farm dwelling," or "mixed" (some subjects having lived on farms part of the time). Veterans were queried regarding details of their service, such as branch of service and length of time spent in a particular service, and the state in which they spent the most time during domestic military service. The names of the three countries in which each subject had had the longest overseas service were obtained, specified by duration of service in each country and characterized by the degree of contact with native civilians. Note was made of any claimed history of lung disease and of any known contact with tuberculous patients or of any military duties involving the care of the sick. A 70-millimeter photofluorogram of the chest of each student was obtained. This was followed by a 14- by 17-inch film if the photofluorogram was considered to be in any way suspicious.

Approximately three quarters (73 per cent) of the 13,386 subjects studied were veterans. A little more than half of the veterans (55 per cent) had served in the army, 37 per cent had been in the navy, while the remaining 8 per cent had served with the marines, coastguard, or merchant marine. All colleges of the University were represented; the largest contingents came from University College (31.1 per cent), Technical School (28.7 per cent), and the School of Business (8.8 per cent); but significant groups were also contributed by the Graduate School (5.0 per cent) and the Medical and Dental Schools (4.2 per cent). The fact that so many of the students were veterans combined with the fact that graduate students were included in the survey resulted in an age distribution considerably higher than would be considered normal; 57.9 per cent were twenty-three years or older, and 28.0 per cent were twenty-six years or older.

Nearly 90 per cent of the students who were surveyed lived in the West North Central states, the majority being residents of Minnesota. Almost every state and territory of the Union was represented. Moreover, since so many of the students were veterans and somewhat older than the average college student, residence histories often tended to be quite complicated. Relatively few were lifetime residents of a single place, and this fact has indubitably lessened somewhat the value of the survey as a means of mapping the areas of endemic histoplasmosis in Minnesota. The facts that 81 per cent of those surveyed characterized their longest residence as "non-farm" residence, while another 13 per cent claimed "mixed" residence, and only 4 per cent were farm dwellers (the remaining 2 per cent being unknown) demonstrates further the weaknesses of this group as a basis of mapping the state. Despite all of this, the map which was created does exhibit some interesting features to be discussed below.

Unfortunately all of the information desired could not be obtained for every student since at least two visits to the survey were required and, in the event of a positive reaction to triple antigen, a third visit was necessary to read the results of the three individual antigens. Some students failed to return for the second visit, and in these instances the reactions to tuberculin and to the triple fungal antigens were not known. Moreover, of the reactors to triple antigen, approximately one person in six failed to return for the third visit.

While it is, of course, impossible to determine the degree to which the men who failed to return for the second or third visits may have differed from those who kept their appointments, it is the belief of the present writers that here, again, the possible biases are not of practical significance. The observation most closely related to fungal antigen status was the result of roentgenograms, and, as it turned out, men who did not return for reading of results differed not at all from the men who did return in respect to the proportion who had abnormal roentgenograms. Thus, of men whose photofluorograms were considered normal, 9.3 per cent failed to return for reading of the reaction to the triple antigen, while for men whose photofluorograms showed calcification, the corresponding figure was 10.1 per cent. For all men with roentgenographic abnormalities of any kind, the non-response was 11.2 per cent.

The situation with regard to the third visit was similar. Of men with normal films who reacted to the triple antigen, 16.8 per cent failed to return for reading of the separate antigens, while the corresponding proportion of non-response for men with pulmonary calcification was 16.9 per cent, and for men with abnormalities of any kind, 14.4 per cent. As will be seen below, the roentgenographic results were very closely related to the fungal antigen results, particularly to the result with histoplasmin; therefore, the lack of association between roentgenography and non-response on the second and third visits convinced the present writers that selection was a factor of negligible importance with respect to the determination of antigenic status.

OBSERVATIONS

The numbers of tests completed are shown in table 1. In 1,287 cases the reactions to triple antigen could not be determined. Among the 12,099 students for whom test results with triple antigen were obtained, 1,178 (9.7 per cent) reacted. Of the fractions of these tested with the separate antigens, 89.9 per cent were sensitive to histoplasmin, 30.0 per cent reacted to blastomycin, and 13.7 per cent to coccidioidin. In order to calculate the proportion of reactors to each of the

TABLE 1

RESULTS OF TESTS WITH FUNGAL ANTIGENS

	Number	Per Cent	Per Cent
Total subjects surveyed	13,386	100.0	—
Tested with triple antigen	12,099	90.4	100.0
Reactors to triple atigen	1,178	—	9.7
Histoplasmin-tested			
Number tested	980	100.0	—
Number of reactors	881	89.9	8.8
Blastomycin-tested			
Number tested	982	100.0	—
Number of reactors	295	30.0	2.9
Coccidioidin-tested			
Number tested	981	100.0	—
Number of reactors	134	13.7	1.3

specific antigens, it was necessary to take account of the fact that approximately 17 per cent of the reactors to the triple antigen were not tested with the separate antigens. Thus, the over-all histoplasmin reactor rate was determined as

$$H = 100 \times \frac{1,178}{12,099} \times \frac{881}{980} = 8.8 \text{ per cent}$$

In this equation, the first fraction represents the proportion of students who reacted to the triple antigen and the second fraction represents the proportion sensitive to histoplasmin among those who reacted to triple antigen and who were tested with the individual antigens. Similarly, the over-all blastomycin reactor rate was 2.9 per cent, while only 1.3 per cent reacted to coccidioidin.

In the tables which follow, those who received triple antigen are shown as "Number Tested." While the exact sampling distribution of reactor rates calculated as described above is necessarily quite complicated, it appears that they may for practical purposes be treated as if they were ordinary percentages based upon denominators approximately nine-tenths as large as the "Number Tested."

For each of the antigens the relative frequency of positive reactors increased rather sharply with increasing age (table 2). Beyond the age range eighteen to

TABLE 2

REACTOR RATES TO THE FUNGAL ANTIGENS BY AGE

Age	Number Tested*	Per Cent of Reactors to		
		Histoplasmin	Blastomycin	Coccidioidin
Total	12,099	8.8	2.9	1.3
Not stated	7	0	0	0
17	35	8.6	0	0
18	641	5.6	1.2	0
19	975	4.9	1.5	0.1
20	1,039	5.5	1.2	0.3
21	1,159	8.4	2.1	1.1
22	1,275	6.4	1.9	0.9
23	1,304	8.0	2.0	1.7
24	1,234	9.0	3.1	1.4
25	1,072	11.5	4.8	1.8
26	866	10.1	4.3	1.7
27	663	10.6	3.7	2.4
28	530	10.8	5.3	2.0
29	360	11.1	3.8	2.4
30	261	15.4	7.7	3.0
31	170	13.3	4.9	2.1
32	150	12.8	5.1	1.7
33	87	19.5	3.3	0
34	69	18.7	6.2	4.7
35–39	139	13.3	5.0	3.3
40–49	63	22.2	1.9	1.9

* With triple antigen

TABLE 3

HISTOPLASMIN REACTOR RATES BY VETERAN STATUS AND AGE

Age	Veterans		Non-Veterans	
	Number Tested*	Per Cent of Reactors	Number Tested*	Per Cent of Reactors
Total	8,833	9.5	3,266	6.7
Not stated	7	0	—	—
17–19	24	0	1,627	5.3
20–22	2,283	6.9	1,190	6.5
23–25	3,407	9.4	203	9.9
26–28	1,957	10.6	102	7.8
29–31	730	12.6	61	18.0
32–34	282	16.1	24	13.9
35 and older	143	15.1	59	18.6

* With triple antigen

thirty-four, the histoplasmin reactor rates increased on the average about 0.7 per cent with each year of age, while the blastomycin and coccidioidin rates increased about 0.30 per cent and 0.15 per cent with each year.

While a higher proportion of veterans than non-veterans reacted to histoplasmin (9.5 per cent *vs.* 6.7 per cent), this was the result of the greater average age of veterans than non-veterans, as may be seen from table 3. Similar results held for the other two fungal antigens. Among veterans, overseas service per se had no influence on the reactor rates; the small differences were attributable to the greater average age of men who served overseas. Similarly, although army veterans had slightly higher reactor rates than did navy veterans (10.6 *vs.* 8.6 for histoplasmin; 3.9 *vs.* 2.8 for blastomycin; 2.2 *vs.* 1.0 for coccidioidin), this finding is explained in large part by the fact that army veterans were, on the average, 1.4 years older than navy veterans. If allowance is made for this age difference, the already small differences in reactor rates among veterans of the different services become trivial.

While the great majority of the students surveyed were residents of Minnesota, there were 13 states which were represented by at least 50 residents among those for whom the results of the triple antigen test were known. The proportion of reactors to the fungal antigens for residents of these 13 states is shown in table 4. Fairly high reactor rates to histoplasmin were found among students who claimed as their states of longest residence, in decreasing order of the reactor rate, Ohio (37.8 per cent); Illinois (31.3 per cent); Nebraska (28.0 per cent); Michigan (19.0 per cent); Iowa (18.4 per cent); and California (15.8 per cent). Relatively high proportions of reactions to blastomycin were found for residents of Nebraska (14.0 per cent) and Illinois (10.2 per cent). Among the 13 states for which there is information, only residents of California exhibited an elevated rate of reaction to coccidioidin (12.6 per cent). It is believed that the fact that the two states characterized by high blastomycin rates (Nebraska and Illinois) are also characterized by relatively high histoplasmin rates is no accident, but is a consequence

TABLE 4

REACTOR RATES TO THE FUNGAL ANTIGENS BY STATE OF LONGEST RESIDENCE

State of Longest Residence	Number Tested*	Per Cent of Reactors to		
		Histoplasmin	Blastomycin	Coccidioidin
New York	94	8.2	5.5	1.4
Pennsylvania	56	8.9	6.7	0
Ohio	45	37.8	8.1	0
Illinois	150	31.3	10.2	0.8
Michigan	63	19.0	3.5	0
Wisconsin	378	6.7	2.4	2.4
Minnesota	9,987	7.3	2.4	1.2
Iowa	244	18.4	6.2	1.1
North Dakota	253	6.2	2.6	1.0
South Dakota	191	7.3	3.4	1.1
Nebraska	50	28.0	14.0	0
Montana	70	7.1	2.4	0
California	74	15.8	0	12.6

* With triple antigen

TABLE 5

REACTOR RATES TO THE FUNGAL ANTIGENS BY HISTORY OF LUNG DISEASE

History of Lung Disease	Number Tested*	Per Cent of Reactors to		
		Histoplasmin	Blastomycin	Coccidioidin
Total	12,099	8.8	2.9	1.3
None	9,701	8.6	2.9	1.3
Tuberculosis	86	16.2	4.6	0
Pneumonia	701	9.8	2.8	1.6
Pleurisy	94	8.2	4.1	4.1
Bronchitis	174	7.4	3.4	0.6
Influenza	51	15.0	8.3	0
Other	128	9.9	2.0	1.0

* With triple antigen

of considerable cross-reaction as between histoplasmin and blastomycin. This subject will be discussed in more detail below.

The results of the fungal antigen tests for students who claimed to have a history of certain lung diseases are shown in table 5. It is striking to observe that men claiming a history of tuberculosis showed a significantly higher proportion of positive reactors to histoplasmin (16.2 per cent) than did men who claimed no history of lung disease (8.6 per cent). This observation, coupled with the fact that only about three-quarters of the men who claimed a history of tuberculosis reacted to tuberculin is strongly suggestive of previous diagnostic inaccuracies. This situation is easily understandable in view of the fact that so many diagnoses of tuberculosis in the past have been based almost entirely upon roentgenographic

TABLE 6

REACTOR RATES TO THE FUNGAL ANTIGENS BY ROENTGENOGRAPHIC INTERPRETATION

Roentgenographic Interpretation	Number Tested*	Per Cent of Reactors to		
		Histoplasmin	Blastomycin	Coccidioidin
Total	12,099	8.8	2.9	1.3
Negative	11,768	8.2	2.7	1.3
Calcification	107	58.5	21.4	2.2
Other abnormalities (without calcification)	154	14.8	4.2	2.1
Roentgenogram not obtained	70	11.4	2.9	0

* With triple antigen

shadows; and calcific deposits have been considered to be evidence of tuberculosis. Actually, it is impossible to distinguish between the lesions of histoplasmosis and those of tuberculosis merely on the basis of the shadows these diseases cast on roentgenograms. The lesions can be reliably distinguished only by the demonstration of the responsible organism or, failing that, sometimes by histoplasmin and tuberculin skin tests.

The finding that men with histories of "influenza," like those with tuberculosis, are more likely than others to exhibit allergy to histoplasmin was a little surprising. The implication is that in some instances histoplasmosis in its benign, nondisseminated form may be attended by symptoms and signs sufficiently like those of influenza to be mistaken for this latter disease. This result is confirmed by the finding of Furcolow, Menges, and Larsh (15), who studied an epidemic of histoplasmosis in a family of five persons and discovered that in all five instances the disease was originally considered to be influenza and, in fact, was so diagnosed by a physician in at least one case.

Of the 12,099 students tested with triple antigen 11,768 (97.3 per cent) had clear chest films (table 6) and, of this subgroup, 8.2 per cent reacted to histoplasmin. On the other hand, 107 men were found to exhibit calcification on roentgenograms, and 58.5 per cent were reactors to histoplasmin. It seems fairly evident, therefore, that approximately one-half of the calcifications seen in roentgenograms represented the residuals of histoplasmosis. It should be noted here that the presence of calcifications was associated with only a small increase in the proportion of reactors to tuberculin: 30 per cent reacted to tuberculin compared with 23 per cent among men with clear films.

It is instructive to view the situation from another standpoint. Of 881 known reactors to histoplasmin, 52 (5.9 per cent) showed calcification on roentgenograms, while, of 10,921 known non-reactors, only 42 (0.4 per cent) showed such calcifications. It may be seen, therefore, that the majority of pulmonary calcifications seen on roentgenograms of young men in this area are attributable to previous infection by *Histoplasma capsulatum* although such calcifications are a relatively infrequent consequence of histoplasmosis, being seen only in approximately one of 17 previously infected persons.

The histoplasmin reactor rates for residents of the different counties of Minnesota are tabulated in table 7. These data were used to construct the map shown in figure 1. Students have been credited only to counties wherein they had at least five years of residence. The majority of students, as has been noted above, did not have simple residence histories so that a restriction to "lifetime" residents of particular counties would have dissipated the data. Even under the rather mild restriction on duration of residence which was employed, some counties are represented only by very few men. Nevertheless, some rather interesting patterns are discernible.

Most striking is the area of relatively high rates (more than 15 per cent reactors) which apparently reaches its peak in Carver County, where the reactor rate is 31.3. Although the data shows Wright County (the southeast corner of which bounds Carver on the north) to be a county of low over-all rate, Smith (11) in an intensive study of histoplasmin sensitivity in Wright, found quite high rates (25 to 30 per cent) in the corner adjacent to Carver. The reactor rate, however, fell off at a very steep gradient in a northwesterly direction. Although our data are too gross to support an investigation of fine detail in the map, it does seem to be true that the focal area of high rates, consisting approximately of Carver, Scott, Le Sueur, and Nicollet counties (figure 1, black area in southeast central part of state) does not represent merely an extension of the larger area of high histoplasmin rates to the south and east of Minnesota as mapped by Furcolow. Rather there seems to be a local peak in the area cited.

The high rates shown for Red Lake and Mahnomen counties are based on only a handful of cases; whether these rates are indeed significantly high or whether the small numbers are deceptive, it is impossible to say. However, in Becker County immediately south of Mahnomen County, Rutledge (10) described an outbreak of histoplasmosis which occurred in 1948. Moreover, Clearwater County immediately east of Red Lake and Mahnomen counties showed 10.5 histoplasmin reactors in our survey (figure 1, dark areas in northwestern part of state).

Cross-reactions among fungal antigens: Many investigators have noted the frequent occurrence of cross-reactions among fungal antigens. More particularly, Emmons and associates (16) and Howell (17), in an admirable series of experiments, demonstrated that there was a considerable degree of cross-reaction in guinea pigs between histoplasmin and blastomycin. Animals tested with the antigen heterologous to the fungus with which they were infected commonly reacted almost as strongly as when tested with the homologous antigen. These results varied with the sensitivity of the animals and more particularly with the lot and dilution of antigen. In any event, reaction to either antigen could not be taken to imply infection with the homologous fungus; certainly not in the absence of careful standardization of the antigen employed. Cross-reactions between coccidioidin and the other two fungal antigens were much less frequent.

In view of these results, it is not surprising to find that, of the 295 blastomycin reactors in the present survey, only 11 did not also react to histoplasmin. Two of the 11 reacted to coccidioidin, so that only 9 of 12,000 persons tested were pure blastomycin reactors. Eight of the 9 were residents of Minnesota, while the ninth

TABLE 7

HISTOPLASMIN REACTOR RATES FOR MINNESOTA COUNTIES

(Includes All Men Having at Least Five Years of Residence in the County)

County	Number Tested*	Per Cent of Reactors	County	Number Tested*	Per Cent of Reactors
Aitkin	44	9.1	Martin	68	7.4
Anoka	79	6.3	Meeker	47	10.6
Becker	42	7.1	Mille Lacs	52	3.9
Beltrami	51	2.0	Morrison	52	5.8
Benton	36	0.0	Mower	81	9.9
Big Stone	27	7.4	Murray	33	3.0
Blue Earth	118	8.5	Nicollet	47	21.3
Brown	61	13.1	Nobles	50	5.3
Carlton	59	4.5	Norman	31	9.7
Carver	48	31.3	Olmsted	120	7.8
Cass	42	4.8	Otter Tail	116	6.0
Chippewa	57	8.8	Pennington	33	3.0
Chisago	64	10.7	Pine	55	7.3
Clay	52	5.8	Pipestone	32	6.3
Clearwater	19	10.5	Polk	88	8.0
Cook	10	0.0	Pope	33	9.1
Cottonwood	35	5.7	Ramsey	1,948	6.8
Crow Wing	91	7.0	St. Paul	1,904	7.0
Dakota	129	10.7	Rest of county	44	2.3
Dodge	30	15.0	Red Lake	8	25.0
			Redwood	67	2.5
Douglas	65	3.1	Renville	64	4.7
Faribault	56	7.1			
Fillmore	60	6.7	Rice	81	14.8
Freeborn	91	11.0	Rock	27	5.6
Goodhue	107	8.0	Roseau	27	7.4
			St. Louis	781	5.1
Grant	24	4.2	Duluth	350	5.6
Hennepin	4,416	7.9	Rest of county	431	4.7
Minneapolis	4,190	7.5	Scott	33	21.2
Rest of county	226	14.5			
Houston	29	13.8	Sherburne	29	6.9
Hubbard	30	6.7	Sibley	45	6.7
Isanti	54	4.9	Stearns	161	5.0
			Steele	50	7.6
Itasca	118	7.4	Stevens	28	7.1
Jackson	40	7.5			
Kanabec	30	13.3	Swift	47	10.2
Kandiyohi	66	6.1	Todd	46	0.0
Kittson	16	0.0	Traverse	23	4.4
			Wabasha	60	5.0
Koochiching	48	6.3	Wadena	32	3.1
Lac Qui Parle	45	4.4			
Lake	22	0.0	Waseca	32	6.3
Lake of the Woods	16	0.0	Washington	95	6.8
Le Sueur	49	20.4	Watonwan	41	9.8
			Wilkin	10	0.0
Lincoln	30	3.3	Winona	83	12.1
Lyon	71	9.9			
McLeod	66	10.6	Wright	67	4.5
Mahnomen	7	42.9	Yellow Medicine	43	0.0
Marshall	32	0.0			

* With triple antigen

TABLE 8

CROSS REACTION BETWEEN HISTOPLASMIN AND BLASTOMYCIN

Histoplasmin Reaction Diameter of Induration	Blastomycin Reaction	
	Number of Reactors	Per Cent of Reactions with Induration of at Least 13 Mm. in Diameter
4 mm. or less (nonreactors)	11	9.1
5–7 mm.	7	0.0
8–12 mm.	124	6.5
13–17 mm.	124	16.1
18 mm. or more	29	31.0

was a resident of New York. All of the 9 were veterans, which is not in itself remarkable, but no other common characteristic was obvious.

In general the reactions to blastomycin were of smaller size than were those to histoplasmin in the same subjects; moreover, the sizes of the blastomycin and histoplasmin reactions were highly correlated. There were 284 men who reacted to both histoplasmin and blastomycin. Of these, the diameter of induration of the blastomycin reaction exceeded that of histoplasmin in only 20 instances, while the histoplasmin reaction was larger than the blastomycin in 178 men. In the remaining 86, indurations were essentially of equal size. The correlations between the sizes of the two reactions may be seen in table 8.

These observations tempt one to conclude that almost all of the blastomycin reactions are attributable to infection with *Histoplasma capsulatum*, but an alternative explanation is possible: that histoplasmin in adequate dosage is fully as capable of eliciting the *Blastomyces* reaction as is blastomycin itself and that, as prepared for use in the present study, histoplasmin is more potent than blastomycin.

On the basis of similar observations in another study, Furcolow and co-workers (18) concluded that "It appears from these findings that the blastomycin reactions are cross-reactions." It seems to us *possible* that as many as a third of the blastomycin reactions observed in the present study resulted from infection by *Blastomyces*.

The situation with respect to cross-reaction between histoplasmin and coccidioidin appears easier to interpret. There were but 134 coccidioidin reactors found. Of these, 68 (51 per cent) also reacted to histoplasmin, while the relationship between the sizes of the two reactions are shown in table 9.

The fact that coccidioidin reactions in the presence of strong histoplasmin reactions tend to be smaller, on the average, than those of coccidioidin elicited in men with little or no histoplasmin sensitivity seems to imply that many of the reactions to coccidioidin which occurred together with histoplasmin of 8 mm. or more were in fact cross-reactions occasioned by infection with *Histoplasma capsulatum*. A converse situation also holds. It appears safe to conclude that many of the histoplasmin reactions observed in men strongly sensitive to coccidioidin represented cross-reactions due to coccidioidal infection (table 10).

TABLE 9

CROSS REACTION BETWEEN HISTOPLASMIN AND COCCIDIOIDIN

Histoplasmin Reaction Diameter of Induration	Coccidioidin Reaction	
	Number of Reactors	Per Cent of Reactions with Induration of at Least 13 Mm. in Diameter
4 mm. or less (nonreactors)	66	31.8
5–7 mm.	12	41.7
8–12 mm.	34	20.6
13–17 mm.	16	12.5
18 mm. or more	6	16.7

TABLE 10

CROSS REACTION BETWEEN COCCIDIOIDIN AND HISTOPLASMIN

Coccidioidin Reaction Diameter of Induration	Histoplasmin Reaction	
	Number of Reactors	Per Cent of Reaction with Induration of at Least 13 Mm. in Diameter
4 mm. or less (nonreactors)	812	41.4
5–7 mm.	17	47.1
8–12 mm.	36	30.6
13–17 mm.	11	18.2
18 mm. or more	4	25.0

TABLE 11

HISTOPLASMIN REACTORS AMONG THOSE STRONGLY SENSITIVE TO COCCIDIOIDIN

State	Histoplasmin Nonreactors		Histoplasmin Strong Reactors	
	Number of Coccidioidin Reactors			
	All Reactors	Strong Reactors	All Reactors	Strong Reactors
California	20	7	4	2
Arizona	15	5	2	1
Texas	13	2	1	0
None of above	18	6	15	0
Total	66	20	22	3

This conclusion is confirmed by an analysis in relation to geography. Attention is directed to the reactors to coccidioidin who failed to react to histoplasmin (66 subjects) and to those who gave strong reactions to histoplasmin (22 subjects) (diameter of induration 13 mm. or more) in order to sharpen the distinction. If we classify men according to whether or not they gave histories of residing (either as civilians or in military service) in a state in which coccidioidomycosis is known to be endemic, the result is seen in table 11.

It is significant that, of the 66 coccidioidin reactors who did not react to histoplasmin, only 18 (27 per cent) claimed no history of residence in a *Coccidioides* endemic area; while, of the 22 who were strong histoplasmin reactors, 15 (68 per cent) were in this category. Moreover, while 20 of 66 (30 per cent) of the histoplasmin nonreactors were strong coccidioidin reactors, only 3 of 22 (14 per cent) of the strong histoplasmin reactors were strong coccidioidin reactors; and all 3 of the strong coccidioidin reactors in this group claimed residence in an endemic area.

Correlations between coccidioidin and blastomycin reactions did not seem to be revealing. Since at least the majority of the blastomycin reactions were cross-reactions to *Histoplasma capsulatum*, very little could be learned by comparing the coccidioidin with the blastomycin readings.

DISCUSSION

A triple fungal antigen which was used in the present survey has been described. To determine the histoplasmin, blastomycin, and coccidioidin reactors in the group of 12,099 subjects, using this technique, 12,099 injections of triple antigen were first required. Since 1,178 subjects reacted to triple antigen, for each of whom three injections of separate antigens had to be used, a total of 3,534 injections of separate antigens were required. Thus, the reactions to all three antigens were determined or would have been, had all of the 1,178 returned for readings of the specific tests for a total cost of 15,633 injections, or approximately 1.3 injections per person. Since the use of separate antigens alone would have required three injections per person, the labor of giving injections (and reading the results) was reduced by about 57 per cent in the present study.

The feasibility of using triple antigen in order to simultaneously detect those who react to any of the three fungal antigens depends upon the twin assumptions that those who react to triple antigen are in fact reactors to at least one of the separate antigens and that those who are reactors to at least one of the separate antigens will react to the triple antigen.

The first of these assumptions is easy to test on the data from the present study. Of 979 men who were positive reactors to triple antigen (at least 5 mm. of induration) and who were tested with individual antigens, only 24 failed to react to any one of the three separate antigens. It seems to the writers, however, that, as these men constituted fewer than 3 per cent of the group of reactors to triple antigen, it may be assumed for practical purposes that the first assumption is justified.

The converse situation is not so clear. As the men were not tested with the separate antigens if they did not react to the triple antigen, it is not possible to tell how many might have reacted to the separate antigens despite nonreaction to the combination. However, the reactions to the triple antigen were generally at least as large as the reactions to the separate antigens in men who reacted to both. For histoplasmin, the single antigen response was larger in 20 per cent, smaller in 34 per cent, and essentially the same in 46 per cent; while for blastomycin and coccidioidin, in only 8 per cent and 13 per cent, respectively, was the

reaction to the single antigen stronger than the reaction to the combination. Moreover, in the minority in whom a larger single antigen response was observed, the difference in diameter of induration rarely exceeded 5 mm. Therefore, although the present data do not prove the point, it seems unlikely, in view of this circumstantial evidence, that men who would have reacted to the separate antigens failed to react to the combined antigen.

The savings which can be made through the use of the device of a combined antigen are dependent upon the proportion of reactors to triple antigen in the group studied. Thus, if 70 per cent (rather than 9.7 per cent) should react to triple antigen, to test a group of 1,000 men would require 1,000 injections of triple plus 2,100 injections of separate antigens, so that an average of 3.1 injections per person would be required (as against 3 for separate antigens) and the technique would be wasteful rather than saving of effort.

Another point to be borne in mind is that three visits are required of each subject if triple antigen is used. In the present study, 17.4 per cent (almost one in six) of those injected with separate antigens failed to return for the third visit. Thus, not only was there loss of information by making the survey dependent upon a third visit, but the task of calculating reactor rates was much complicated by the necessity of having constantly to take account of the group for whom only partial information was available, i.e., those known to react to triple antigen, but unknown with respect to separate antigens.

A further question to be considered relates to the advisability of testing groups with the blastomycin presently available. The consensus is that at least a majority are cross-reactions. In any event, whatever mechanism is at work, in the present study, 96.3 per cent of the men who reacted to blastomycin also reacted to histoplasmin, and the information with respect to blastomycin sensitivity was practically valueless except as it related to the subject of histoplasmin—blastomycin cross-reactions. The general use of blastomycin in surveys would not be recommended until such time as a much more specific antigen is available except possibly in areas wherein the incidence of histoplasmosis infection is known to be extremely low.

If only two fungal antigens were to be used—say histoplasmin and coccidioidin—the economics of the problem would change considerably. In such a situation, only two injections per person would be required if separate antigens were used from the start. If a double antigen were used, to be followed by separate antigens for reactors to the double antigen, the number of injections per person required on the average would, as before, depend upon the reactor rate to the combined antigen. If this were 10 per cent, an average of 1.2 injections would be required for a saving of 40 per cent in the number of injections and readings. However, if 50 per cent reacted to the double antigen, then the costs would be equal for the two systems. If the combined antigen reactor rate exceeded 50 per cent, costs would be lower if separate antigens were used from the start.

All in all, the present writers are unable to recommend—and indeed would advise against—use of combined antigen unless the combined antigen reactor rate is known to be fairly low, not more than 15 per cent (this may be determined in a pilot study).

SUMMARY

A survey of reactions to histoplasmin, blastomycin, and coccidioidin was made on 12,099 students at the University of Minnesota.

Cross-reactions between histoplasmin and blastomycin were so numerous that the blastomycin reactor rates were not meaningful. Most of the calcifications seen on roentgenograms were attributable to previous infection with *Histoplasma capsulatum*. The findings of this survey and more frequent reporting of clinical cases indicated that the incidence of histoplasmosis in Minnesota is higher than had been anticipated. Many who claimed histories of tuberculosis and influenza probably had previous histoplasmosis.

Military service, per se, had no noticeable over-all effect on reactor rates. However, a few men who had served in *Coccidioides* endemic areas reacted to coccidioidin. A map of the *Histoplasma* endemic areas of Minnesota is presented.

Although some economic savings can be effected by using multiple fungal antigen followed by separate tests of the reactors, the attendant difficulties resulting from the necessity of three visits go far towards counter-balancing the advantages.

SUMARIO

Sensibilidad a los Antigenos de Hongos entre los Estudiantes de la Universidad de Minnesota

En 12,099 estudiantes de la Universidad de Minnesota se llevó a cabo una indagación de las reacciones a la histoplasmina, la blastomicina y la coccidioidina.

Tan numerosas fueron las reacciones cruzadas entre la histoplasmina y la blastomicina que carecen de significado los índices de reactores a la última. La mayor parte de las calcificaciones observadas en las radiografías eran imputables a infección anterior por *Histoplasma capsulatum*. Los hallazgos de esta encuesta y la denuncia más frecuente de casos clínicos indicaron que la incidencia de la histoplasmosis en Minnesota es más alta de lo esperado. Muchos que alegaron historias de tuberculosis y de influenza habían tenido probablemente histoplasmosis antes.

El servicio militar, por sí, no ejerció efecto global apreciable sobre los índices de reactores. Sin embargo, algunos sujetos que habían servido en zonas de endemicidad del *Coccidioides* reaccionaron a la coccidioidina. Preséntase un mapa de las zonas de Minnesota en que el *Histoplasma* es endémico.

Aunque pueden hacerse algunos ahorros económicos usando antígeno múltiple de hongos, seguido de pruebas separadas de los reactores, las dificultades dimanantes de la necesidad de hacer tres visitas van muy lejos en contrapesar las ventajas.

RESUME

Sensibilité aux antigènes fongiques chez des étudiants de l'Université de Minnesota

Une enquête sur les réactions à l'histoplasmine, la blastomycine et la coccidioïdine a été effectuée chez 12.099 étudiants de l'Université de Minnesota.

Les réaction croisées entre l'histoplasmine et la blastomycine étaient si nombreuses que les taux de réacteurs à la blastomycine n'étaient pas significatifs. La plupart des calcifications observées sur les radiogrammes étaient imputables à une infection antérieure par l'*Histoplasma capsulatum*. Les résultats de cette enquête et la publication plus fréquente d'observations de cas cliniques indiquèrent que l'incidence de l'histoplasmose dans le Minnesota était plus élevée qu'on ne le supposait. Un grand nombre de ceux qui accusaient des antécédents de tuberculose et d'influenza avaient probablement été antérieurement atteints d'histoplasmose.

Le service militaire, en soi, n'affectait pas de façon particulière le taux de réacteurs.

Toutefois, quelques hommes ayant servi dans les secteurs où la *Coccidiose* était endémique réagissaient à la coccidioïdine. Une carte des régions du Minnesota où l'*Histoplasma* est endémique est présentée.

En dépit d'une certaine économie réalisable par l'emploi d'un multi-antigène fongique suivi par des tests séparés chez les réacteurs, les complications entrainées par la nécessité de trois visites dépassent de beaucoup les avantages.

Acknowledgment

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ANTIGENIC RELATIONSHIPS BETWEEN MYCOBACTERIA AS DETERMINED BY AGAR DIFFUSION PRECIPITIN TECHNIQUES[1,2]

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INTRODUCTION

The *Mycobacteriaceae* comprise a family of microorganisms which for lack of more definitive criteria are classified principally on the basis of their morphology and their virulence for experimental animals.

Attempts at classification by serologic methods have been unrewarding because of the possession of common antigens by various members of this family and because of the difficulties encountered in the isolation and purification of antigenic substances. Seibert and co-workers (1–5), Heidelberger and Menzel (6–10), McCarter and Bevilacqua (11), Heckley and Watson (12, 13), Creighton and associates (14–16), Vellick (17), and Coghill (18) have attempted to isolate chemically specific antigens from mycobacteria but have been unable to obtain a series of antigens with which a serologic classification could be established.

Attempts to serologically differentiate mycobacterial strains by such reactions as the precipitation, agglutination, hemagglutination, and complement-fixation tests, using a variety of crude antigens such as cell extracts, Old Tuberculin, and whole cells (heat-killed or viable), have been undertaken by such investigators as Mudd and Smith (19), Gaby, Black, and Bondi (20), Tullock, Munro, Ross, and Cummings (21), Rice and Reed (22), and Wilson (23). While many of these attempts have contributed interesting immunologic data, none of them has demonstrated a way to fulfill the broader goal of achieving a satisfactory serologic differentiation of these organisms.

The isolation of many atypical strains of mycobacteria from patients with disease conditions which resemble tuberculosis emphasizes the need for new criteria to be used in the identification and classification of these organisms. A serologic method which would differentiate between the component antigens present in extracts or culture filtrates of mycobacterial cells and show the antigenic relationships between those found in different mycobacteria would be of great value. The Oudin diffusion precipitation technique is a serologic method which has been used successfully in the study of such antigenic complexes as serum proteins by Jager (24); egg albumins by Becker (25) and by Deutsch (26); and streptococcal antigens by Jennings and Malone (27, 28); and the detection of even minute antigenic differences which exist between the larval, pupal, and adult forms of the Cecropia silk worm by Telfer (29).

In view of the fact that these complex antigen mixtures have been successfully analyzed by agar diffusion methods, it seemed that some of these techniques

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[2] Aided by a research grant from Parke, Davis & Company, Detroit, Michigan.

might be applied to the determination of the antigenic composition of acid-fast microorganisms. To demonstrate that these techniques are adaptable to this purpose is the major theme of this presentation.

METHODS AND MATERIALS

Culture filtrate antigen preparation: Strains of mycobacteria employed as sources of antigen were grown as pellicle cultures on modified Proskauer and Beck medium (30) at 37°C. for one to sixteen weeks. The cultures were then passed through sintered glass filters to free the medium of cells and debris. Initial experiments revealed that concentration of these filtrates was necessary to achieve satisfactory antigen preparations, since a high antigen concentration is required in the Oudin system to ensure a relatively constant supply of antigen to the antibody incorporated in the agar column. Concentration was carried out through pervaporation as outlined by Jennings (27) and resulted in reduction of volume to 1 to 5 per cent of the initial volume. Lyophilization, in lieu of pervaporation, failed to yield a more satisfactory product. Unmetabolized media components, concentrated simultaneously with the antigenic substances, were found experimentally not to interfere with the precipitation reactions.

In pilot experiments, using both the Oudin and the modified Ouchterlony tests, cell extracts prepared by the method described by Millman and Youmans (31) were compared with reactions obtained in tests using culture filtrate antigens. The reactions with both preparations were qualitatively and quantitatively equal in the modified Ouchterlony test. Due to the cloudy appearance and a tendency toward sedimentation of the cell extracts in the Oudin tests, the culture filtrate preparations proved to be better; therefore, culture filtrate preparations were used throughout this investigation.

Preparation of antisera: Antisera were produced through subcutaneous inoculation of white rabbits with culture filtrates incorporated in Freund's adjuvant (32). Weekly injections were given over a period of six weeks, with a time lapse of ten to twelve days following the last injection before intracardial exanguination was undertaken. Serum was harvested by routine methods, filtered, and stored at 4°C. until needed.

Mycobacterial strains: A complete list of the mycobacterial cultures used is given in table 1.

The Oudin simple diffusion method: Simple diffusion determinations in agar depend upon the diffusion of a concentrated, soluble antigen preparation through a column of agar gel which contains antiserum. As antigen diffusion proceeds, a continuum of decreasing antigen concentration develops from the antigen agar interface downward. At some point along this continuum each component of the antigen mixture will achieve a concentration relative to its specific antibody which is within the equivalence zone, and a visible ring of precipitate will develop. Each individual antigen in the mixture diffuses independently and at a rate determined by the concentration of the antigen, the molecular shape and weight of the antigen, and the concentration of the antibody in the agar column. Therefore, the equivalence ratios of antigen to antibody, for each system, occur at various levels in the agar column and result in a visible separation of the precipitate rings formed by the different antigen-antibody systems.

Individual Oudin tests were routinely prepared in triplicate and were controlled by the simultaneous use of reaction tubes which contained normal rabbit serum as well as antibody agar tubes overlayered with both undiluted and a 1 per cent dilution of Old Tuberculin. Precipitates were never observed in these controls.

Incubation of the Oudin tubes was carried out at 37°C. in a hot air incubator. Strict temperature control was exercised in order to prevent what Becker (25) refers to as nonmigrating temperature artifact bands.

The modified double diffusion technique: The method used for the preparation of the double diffusion plates was a simple modification of that described by Jennings and Malone (28). The principles involved in the double diffusion reactions are discussed in detail by Oudin (33).

TABLE 1

SOURCE OF MYCOBACTERIAL ANTIGENS

Mycobacterial Strains	Used for Antiserum Production	Used for Diffusion Tests
I. *Mycobacterium tuberculosis* var. *hominis*, strains:*		
H37Rv	X	X
H37Ra	X	X
R1Rv	X	X
JH6	X	X
VA # 398		X
VA # 464		X
VA # 485		X
VA # 486		X
VA # 499		X
H37 (Phipps)		X
II. *Mycobacterium tuberculosis* var. *bovis*, strains:		
# 4228-2		X
# 4228-4	X	X
Ravenel		X
BCG-1	X	X
BCG-2		X
BCG-3		X
BCG-4	X	X
BCG-5		X
III. *Mycobacterium avium*, strains:		
Sheard	X	X
Kirchberg	X	X
IV. *Mycobacterium:*		
607	X	X
smegmatis	X	X
ranae	X	X
phlei	X	X
vole		X
butyricum		X
V. Atypical acid-fast organisms, strains:†		
# 3		X
# 35G	X	X
# 72C		X
# 80G	X	X
# 118		X
# 123		X
# 160		X
# 170		X
# 171		X
# 182		X
# 198		X
# 212		X
# 217		X
# 222	X	X
# 224		X
# 225		X
# 227		X

* Cultures prefixed with V.A. were recently isolated by Dr. Nelda Holmgren from tuberculous patients at the Chicago Veterans Administration Research Hospital, 300 E. Huron Street, Chicago, Illinois.

† Obtained from Dr. Ernest Runyon, Veterans Administration Hospital, Sunmount, New York.

All BCG strains, H37 (Phipps), and Ravenel were obtained from Dr. S. Rosenthal, Tice Clinic, Chicago, Illinois.

All other strains were supplied by Dr. W. Steenken, Jr., Trudeau Laboratory, Trudeau, New York.

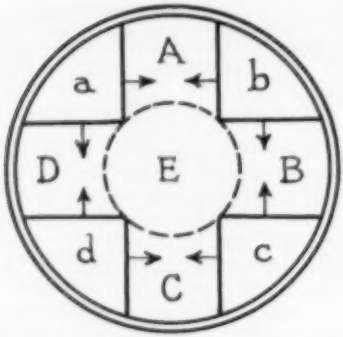

FIG. 1. A schematic representation of the modified Ouchterlony reaction plate, in which *a*, *b*, *c*, and *d* designate reagent wells cut and removed from a solidified agar base. *A*, *B*, *C*, and *D* designate reaction fields in which the precipitation reactions occur subsequent to diffusion of reagents out of wells *a*, *b*, *c*, and *d* in the directions indicated by the small arrows. Reaction field *E* is the area in which confluency of precipitate bands developing in different systems is observed to occur.

Pyrex Petri dishes (5 by 1.5 cm.) were poured with 10 ml. of molten 1.5 per cent agar prepared with 0.8 per cent saline. Upon solidification of the base layer, four quadrants were cut from each plate. Agar reagent dilutions were prepared in a ratio of three parts of reagent to one part of triple-strength saline agar. One milliliter of antibody dilution was pipetted into well (*a*), 1 ml. of homologous antigen dilution into well (*b*). To well (*c*) was added 1 ml. of a heterologous antiserum dilution and to well (*d*) was added 1 ml. of antigen dilution which was homologous to well (*c*) and heterologous to (*a*). This arrangement is clearly visualized in figure 1.

RESULTS

Oudin simple diffusion tests: From a series of determinations in which such variables as reagent concentration, incubation temperature, agar concentration, and length of incubation were altered, it was concluded that a minimum of four antigen-antibody complexes were demonstrable with antigens from human strains of mycobacteria and that this minimum might well constitute the actual total of antigens detectable by this method. Representative data from three human strains tested for homologous and heterologous reactions by the use of culture filtrate preparations and rabbit antisera are given in table 2.

At regular intervals during incubation, measurements of the distance from the meniscus to the diffusion front were made by means of vernier calipers, and mean values so obtained from twenty separate determinations were plotted for each culture filtrate and antiserum system. When the distance of migration (in millimeters) was plotted against the square root of time (in hours), graphic representation of the migration rates of the various complexes within the system was obtained. As an example of such a plot, figure 2 is presented and represents the rates of migration of four different antigen-antibody complexes which appear when H37Ra culture filtrate is reacted with the homologous antiserum.

TABLE 2

NUMBER OF PRECIPITATE BANDS OBSERVED IN 20 OUDIN ANALYSES USING THREE STRAINS OF *MYCOBACTERIUM TUBERCULOSIS* VAR. *HOMINIS* AND OLD TUBERCULIN (OT)

Culture Filtrate Antigens	Rabbit Sera			
	Anti-H37Rv	Anti-H37Ra	Anti-R1Rv	Normal
H37Rv	4	4	4	0
H37Ra	4	4	4	0
R1Rv	4	4	4	0
OT Control Antigens				
OT (undiluted)	0	0	0	0
OT (diluted to 1 per cent)	0	0	0	0

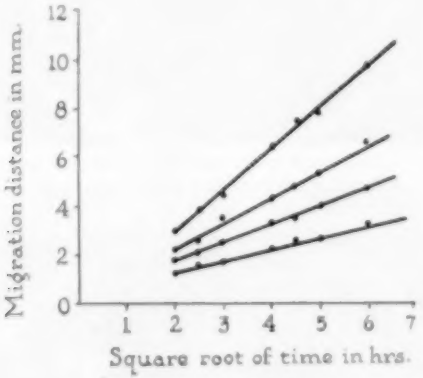

FIG. 2. A graphic representation of the rates of migration of each of the four antigen-antibody complexes observed in the Oudin test by use of H37Ra culture filtrate preparations and homologous rabbit antiserum.

Studies in which the modified Ouchterlony method was used indicated that this method was superior to the Oudin for the purpose of determining antigenic patterns of the mycobacteria. To illustrate this contention, attention is called to figure 3*A*, wherein the homologous and heterologous reactions between antigen prepared from strain 607 and *Mycobacterium smegmatis* are shown. The antigen designated as 607d is a dialyzed and concentrated preparation which was compared for reactivity with a concentrated, undialyzed preparation designated as 607. The designations SM and 607 refer respectively to anti-smegmatis and anti-607 rabbit sera. From the reactions in this photograph it can be observed that the homologous reactions between antigen preparations 607 and 607d and antiserum 607 produce four separate bands of precipitation which are quantitatively equal, as judged from density, and qualitatively equal as is shown

FIGURE 3

Typical precipitation reactions demonstrated by four modified Ouchterlony plates.

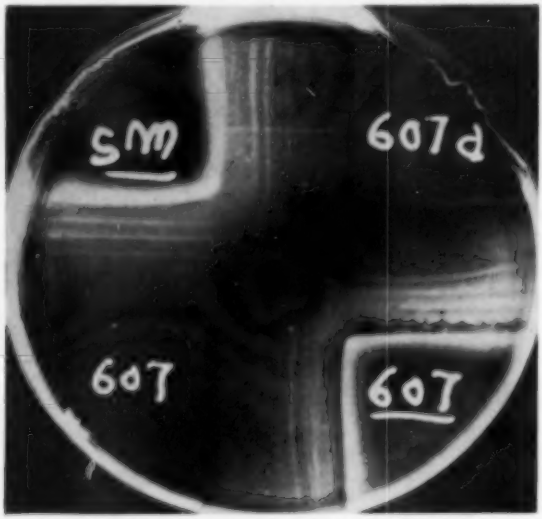

FIG. 3A. 607 antigens designated as 607 and 607d (dialyzed and concentrated 607 antigen) reacted with anti-smegmatis rabbit serum (SM) and anti-607 rabbit serum (607).

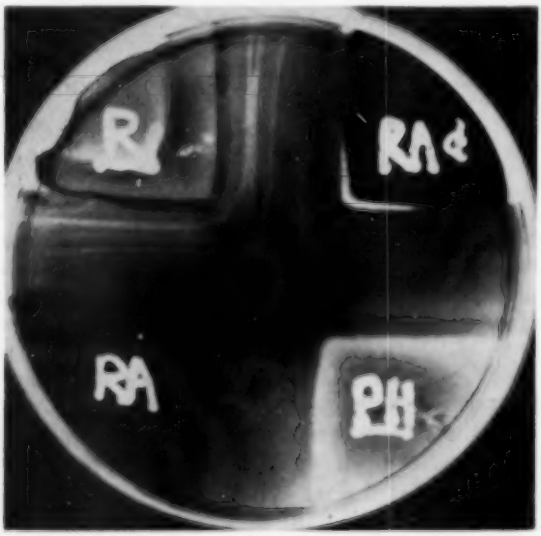

FIG. 3B. Dialyzed and undialyzed culture filtrates from strain H37Ra (respectively designated as RAd and RA) reacted with anti-RIRv serum (RI) and anti-phlei serum (PH).

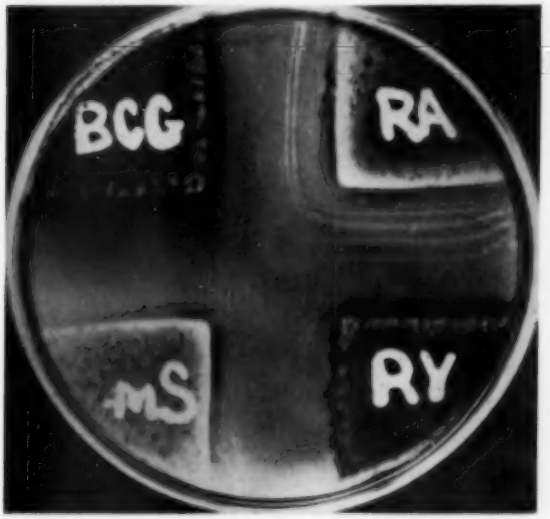

FIG. 3C. BCG-1 culture filtrate antigen (BCG) and H37Rv culture filtrate antigen (RV) reacted with anti-H37Ra (RA) and anti-smegmatis (MS) rabbit sera.

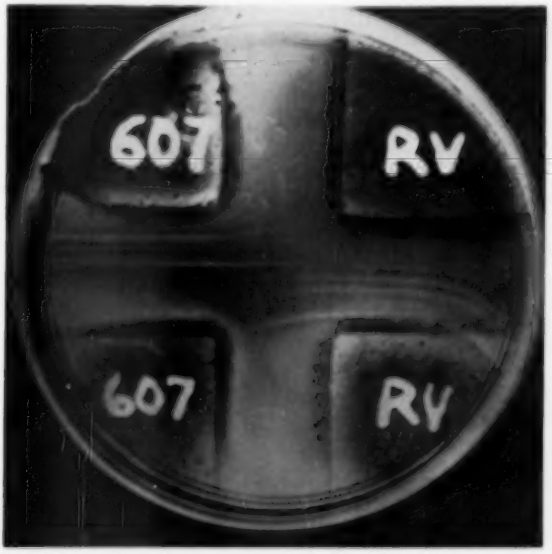

FIG. 3D. H37Rv culture filtrate antigen and the homologous antiserum with 607 culture filtrate antigen and its homologous rabbit antiserum.

by the confluency of the precipitate zones in the reaction field *E* (previously designated in figure 1). The reactions which are shown between antigen preparations 607 and 607d and antiserum SM demonstrate the complete cross reactivity which occurs between 607 and smegmatis.

Additional evidence of a similar cross reaction between H37Ra dialyzed and undialyzed, concentrated culture filtrates (designated accordingly as RA and RAd), and antiserum R1Rv (designated as R1) is shown in figure 3*B*. In this same figure, the absence of any evidence of precipitation in the reaction fields between the two antigen preparations and the anti-phlei rabbit serum (designated as PH is interpreted as indicating the complete failure of cross reactions between the strain phlei antiserum and the two antigen preparations from H37Ra strain to occur.

Figure 3*C* presents reactions between H37Ra antiserum (designated as RA) and BCG-1 and H37Rv concentrated, undialyzed antigens (BCG and RV). In these reactions, confluency of the two zones produced by the BCG-1 antigen and the H37Ra antiserum with two of a total of four zones, produced by the reaction of H37Ra antiserum and the H37Rv antigen indicates the identity between the two BCG antigens and two of the four H37Rv antigens. There is also evidence of failure of cross reaction to appear between the smegmatis antiserum (MS) and the two human strain culture filtrate antigen preparations.

On the other hand, in figure 3*D* are shown the reactions of four bands each, between 607 antigen and homologous antiserum and between H37Rv antigen and homologous antiserum. The absence of cross reactions between these two strains is evidenced both by the failure of precipitation to develop in the reaction fields *A* and *C*, as designated in figure 1, and by the failure of confluency to occur in reaction field *E*.

Table 3 is a composite of all data derived from double diffusion analysis of various mycobacterial strains screened for their antigenic patterns by this method. This table illustrates several interesting antigenic relationships.

It appears that the strains are divisible serologically into four groups arbitrarily designated as *I*, *II*, *III*, and *IV*. *Group I* consists of five human mycobacterial strains, twenty atypical human strains, the avian strain *Kirchberg*, the bovine strain *BCG-4*, and the vole bacillus. There were no reactions between *Group I* antigen preparations and anti-*Group III* and *IV* sera. *Group II*, with the exception of avian strain *Sheard*, is composed of bovine strains only and showed only two antigen-antibody complexes when reacted with both anti-*Group I* and anti-*Group II* sera. As was true of *Group I*, no reaction developed between *Group II* antigens and anti-*Group III* and *IV* sera. *Group III* constitutes a collection of saprophytic strains of mycobacteria and, like *Group I*, possessed a total of four antigenic components none of which gave cross reactions with anti-*Group I*, *II*, or *IV* sera. The antigens demonstrated with these strains appear to be possessed exclusively by *Group III*. *Group IV* consists of a single, atypical chromogenic acid-fast organism whose characteristics are such that it is not possible at this time to conclusively identify this organism as a mycobacterium.

TABLE 3

ANTIGENIC RELATIONSHIPS BETWEEN 42 STRAINS OF MYCOBACTERIA AS SHOWN BY THE NUMBER OF BANDS OF PRECIPITATE WHICH DEVELOPED IN MODIFIED OUCHTERLONY TEST PLATES

Culture Filtrate Antigens			Rabbit Antisera														
			H37Rv	H37Ra	R1Rv	BCG-1	BCG-4	4228-4	*Smegmatis*	607	*Ranae*	*Phlei*	Kirchberg	Sheard	35G	80G	222
H37Rv	4228-2																
H37Ra	4228-4																
R1Rv	Ravenel																
H37(Phipps)	BCG-1	II	2	2	2	2	2	2	0	0	0	0	2	2	0	2	2
JH6	BCG-2																
VA # 398	BCG-3																
VA # 464	BCG-5																
VA # 485	Sheard																
VA # 486																	
VA # 499	.....	I	4	4	4	4	4	4	0	0	0	0	4	2	0	4	4
BCG-4																	
Kirchberg	607																
Vole	*Smegmatis*																
72C	*Ranae*																
80G	*Phlei*	III	0	0	0	0	0	0	4	4	4	4	0	0	0	0	0
118	*Butyricum*																
123																	
160																	
170	35G	IV	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
171																	
182																	
198																	
212																	
217																	
222																	
224																	
225																	
227																	

DISCUSSION

As a mycobacterial culture grows, a wide variety of metabolic and autolytic products are released into the medium, resulting in a chemical mixture of widely varied composition. Such variation in the preparations used as diffusion test antigens tended to complicate the analysis of the results. Briefly outlined, the major complications were as follows:

With rabbit antisera, excess antibody is evidenced in the double diffusion reactions by a "feathering" of precipitate from the dense band toward the source of antibody. This precipitate is capable of masking other precipitate bands, produced by minor antigens, which develop in the area of "feathering"; therefore, it often makes difficult the detection of the actual total of antigen-antibody complexes present.

Antigens also may be present in very low concentration. If such is the case, by means of antiserum dilution, such as described by Becker (25), trace antigens present in the Oudin system may sometimes be revealed. All attempts to apply this method failed to reveal such trace substances in the antigen mixtures.

An antigen also may occur in concentrations markedly in excess of that of others present and therefore produce inhibition of the reaction at all concentration levels achieved in the test.

On a theoretical basis it would be expected that all strains with a common origin would possess similar antigenic patterns. This theoretical expectation was not upheld by the BCG-4 strain culture filtrate, which consistently produced a total of four antigen-antibody complexes in the modified Ouchterlony test, in contrast to a total of only two antigen-antibody complexes produced by each of the other bovine strain antigens (BCG-1, BCG-2, BCG-3, BCG-5, 4228-2, 4228-4, and Ravenel) similarly tested. In addition, when the BCG-4 antigens showed complete cross reactivity of four bands of precipitate with anti-human strain sera, the latter group of bovine strain antigens only cross reacted with two of the four antibodies present in the anti-human sera. This observed difference between the BCG-4 strain and related bovine strains was a consistent finding and may possibly be explained on the basis of either the second or third complication discussed above. With reference to table 3, similar differences are shown to exist between the antigen preparations from the two representative strains of *Mycobacterium avium*.

In spite of the difficulties outlined above, it is clear that a constant number of antigens can be demonstrated in culture filtrates of various strains of acid-fast organisms. Clear-cut demonstrations of differences in antigenic composition are seen when the reactions of the human-bovine group are compared to reactions obtained with the reagents from the known saprophytes, since cross reactions failed to occur between these two groups. Conversely, there is strong indication that the so-called atypical strains are closely related to the human strains since there was complete cross reactivity between the antigens and antisera of these two groups. That such relationships are demonstrable by the methods used opens a broad field for future investigation which need not be restricted to antigen preparations like those used in this study. Progress has already been made in the use of growing cultures of mycobacteria as sources of diffusing antigen for both simple and double diffusion tests. Such tests possibly may be more suitable for the detection of antigens and, in addition, may serve as a more practical method for the serologic identification of mycobacteria in the diagnostic laboratory. Determinations of the immunologic status of the various chemical isolates prepared by Seibert and associates (3), Heidelberger and Menzel (8–10), and others, using the diffusion methods, might also prove to be extremely enlightening.

In conclusion, although it is well recognized that the data may not completely define the antigen structure of the cultures studied, a positive step has been taken toward an understanding of the antigenic relationships between the myco-

bacteria. For the first time, a visualization of the antigenic patterns of some strains has been made and, with further work directed toward perfection of the method and toward standardization of the antigen preparations, the goal of establishing a serologic classification of acid-fast organisms seems more nearly accessible.

SUMMARY

Antigenic relationships between forty-two strains of mycobacteria were investigated by means of the Oudin and a modified Ouchterlony method by the use of culture filtrates as antigens. These culture filtrate analyses revealed that the forty-two mycobacterial strains were divisible into four antigenic groups designated as *I*, *II*, *III*, and *IV*.

Group I comprised the largest group and contained mycobacteria whose culture filtrates contained four distinct antigens capable of precipitating antibody in homologous sera. The identical four antigens were produced by each strain in this group. Member strains of *Group I* were the known human strains (both virulent and avirulent), all but one of a group of atypical acid-fast organisms isolated from human patients, one attenuated bovine strain, and one strain of *Mycobacterium avium*.

Group II consisted mainly of mycobacterial strains of bovine origin and one avian strain. The members of this group produced only two antigens, both of which were identical to two of those which appeared in *Group I* culture filtrates.

Group III was comprised mainly of saprophytic mycobacterial strains. These organisms produced four antigens which were identical from strain to strain within this group but entirely different from antigens of *Groups I*, *II*, and *IV*.

Group IV consisted of a single strain of an atypical acid-fast organism which produced two antigens unrelated to those produced by the organisms of *Groups I*, *II*, and *III*.

SUMARIO

Relaciones Antigénicas entre Micobacterias Determinadas por Técnicas de Difusión de Precipitinas en Agar

Se investigaron las relaciones antigénicas entre 42 cepas de micobacterias por medio del método de Oudin y de una modificación de la técnica de Ouchterlony usando filtrados de cultivos como antígenos. Los análisis de estos filtrados revelaron que las 42 cepas micobacterianas eran divisibles en cuatro grupos antigénicos denominados *I*, *II*, *III* y *IV*.

El *Grupo I* comprendía el número mayor y contenía micobacterias cuyos filtrados culturales contenían cuatro distintos antígenos capaces de precipitar anticuerpo en sueros homólogos. Los mismos cuatro antígenos fueron producidos por cada cepa de este grupo. Las cepas representadas en el *Grupo I* eran las humanas conocidas (virulentas a la par que avirulentas), todas, menos una, de las de un grupo de microbios ácidorresistentes atípicos aislados de sujectos humanos, una cepa bovina atenuada y una cepa del *Mycobacterium avium*.

El *Grupo II* constaba nayormente de cepas micobacterianas de procedencia bovina y de una cepa aviaria. Los miembros de este grupo no produjeron más que dos antígenos, ambos idénticos a dos de los que aparecieron en los filtrados de cultivos del *Grupo I*.

El *Grupo III* estaba constituído principalmente por cepas micobacterianas saprófitas. Estos gérmenes produjeron cuatro antígenos que eran idénticos de cepa en cepa dentro del grupo, pero absolutamente distintos de los antígenos de los *Grupos I*, *II* y *IV*.

El *Grupo IV* constaba de una sola cepa de un microbio ácidorresistente atípico que produjo dos antígenos sin relación alguna con los producidos por los gérmenes de los *Grupos I, II y III*.

RESUME

Relations antigéniques entre les mycobactéries déterminées par les procédés de précipitation et la diffusion dans la gélose

Les relations antigéniques entre 42 souches de mycobactéries ont été étudiées à l'aide de la méthode d'Oudin et de la méthode d'Ouchterlony modifiée en utilisant comme antigènes des filtrats de cultures. L'analyse de ces filtrats de cultures révélèrent que ces 42 souches étaient divisibles en quatre groupes antigéniques qui furent dénommés *Groupes I, II, III, IV*.

Groupe I: était le plus grand et comprenait les mycobactéries dont les filtrats de culture renfermaient quatre antigènes distincts ayant la propriété de précipiter l'anticorps dans les sérums homogènes. Les quatre antigènes identiques étaient produits par chacune des espèces de ce groupe. Les souches faisant partie du *Groupe I* étaient des souches humaines connues (virulentes et avirulentes), à l'exception d'une souche appartenant à un groupe de germes acido-résistants isolés chez des malades, d'une souche atténuée d'origine bovine et d'une souche de *M. avium*.

Groupe II: consistait surtout en souches mycobactériennes d'origine bovine il y avait aussi une souche aviaire. Les membres de ce groupe ne produisaient que deux antigènes, ils étaient tous deux semblables à ceux rencontrés dans les filtrats de culture du *Groupe I*.

Groupe III: se composait principalement de souches mycobactériennes saprophytes d'origine bovine. Ces germes produisaient quatre antigènes qui étaient identiques de souche en souche dans le groupe, mais qui différaient absolument des antigènes des *Groupes I, II, et IV*.

Groupe IV: consistait en une seule souche d'un germe acido-résistant atypique, qui produisait deux antigènes sans relation avec ceux produits par les germes des *Groupes I, II, et III*.

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THE PATHOGENIC BEHAVIOR OF MYCOBACTERIUM ULCERANS AND MYCOBACTERIUM BALNEI IN THE MOUSE AND THE DEVELOPING CHICK EMBRYO[1]

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Introduction

During the last decade two new mycobacteria pathogenic for man have been described: *Mycobacterium ulcerans* (1) and *Mycobacterium balnei* (2, 3). Both cause ulceration of the skin in human patients without involvement of the internal organs. Neither causes tuberculosis on inoculation into guinea pigs, and neither grows on media suitable for tubercle bacilli when incubated at 37°C., but both grow on such media when the incubator temperature is reduced to 33°C.

In an earlier paper from this laboratory (4), the cultural behavior of *M. ulcerans* and *M. balnei* was compared with that of other pathogenic and saprophytic mycobacteria. The results with *M. balnei* confirmed and in part extended those reported by Linell and Nordén (3). The present paper records the results of investigations into the pathogenic behavior of the two mycobacteria in mice and chick embryos.

Materials

Bacterial strains: The origin of most of the bacterial strains employed has been presented in an earlier paper (4). For certain experiments the following additional strains were used: *M. tuberculosis* var. *muris* strain OV164,[2] and *M. ranae*.[3]

Mice: The mice used for the earlier experiments were bred at the Walter and Eliza Hall Institute of Medical Research. The stock, which is multicolored, has been maintained by outbreeding between mice of different colors and parentage. Six- to eight-week-old mice, usually males, were used. Later a subline of this stock, maintained in the Australian National University Animal Breeding Establishment, was used.

Methods

Bacteria were prepared for inoculation by growth in liquid Tween®-albumin medium (5) for appropriate periods. All strains except *M. ulcerans* and *M. balnei* were incubated at 37°C.; the latter were incubated at 33°C. Dispersed growth suitable for animal inoculation was obtained with all strains in this medium although, with *M. phlei*, it was necessary to increase the concentration of Tween® 80 to 0.5 per cent. Inocula were standardized by total counts, carried out in a Petroff-Hauser counting chamber with phase contrast illumination, and viable counts were carried out on oleic-acid-albumin-agar solid media (6).

Experimental Results

Pathogenicity for mice of Mycobacterium ulcerans

MacCallum and associates (1) reported that the outstanding feature of *M. ulcerans* infection of mice, inoculated by the intraperitoneal route, was gross

[1] From the Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia.

[2] Kindly provided by the Commonwealth Serum Laboratories, Melbourne, Australia.

[3] Kindly provided by Dr. René J. Dubos.

edema of the subcutaneous tissues of the whole body which first appears three to six months after inoculation and is occasionally accompanied by ulceration of the scrotum.

In preliminary experiments in the present study, it was found that swelling of the footpad of mice developed within a few days of the inoculation of large doses of *M. ulcerans* into that site. Titrations of culture suspensions in this manner, together with comparisons of the reactions of mice to inoculation by a variety of routes, were therefore performed.

Six-week-old male mice were inoculated in the left hind footpad (0.05 ml. of inoculum) intravenously (0.1 ml.), intracerebrally (0.03 ml.), intranasally (0.05 ml.), and intraperitoneally (0.1 ml.) with various dilutions of a four-week-old Tween®-albumin culture of *M. ulcerans* strain R8, which contained 6×10^7 viable bacterial units per ml. Tenfold dilutions from undiluted to 10^{-7} were used for pad inoculation, and hundredfold dilutions from undiluted to 10^{-4} for the other routes of inoculation. The mice were examined at weekly intervals. The results are summarized in table 1.

Footpad inoculation: The results after pad inoculation were similar in all respects to those obtained by the writer with *M. ulcerans* strain SF at the Rockefeller Institute for Medical Research, New York, in 1949. The feet of inoculated mice became swollen and red at varying intervals after inoculation, and the swelling was slowly progressive, so that the feet became greatly enlarged three or four weeks after swelling was first detected (figure 1). Ulceration then occurred. Unless the affected appendage sloughed off, healing was rare and the mouse retained a greatly swollen ulcerated foot, often complicated by secondary infection.

Swelling of the foot appeared within a week of the inoculation of a large dose of bacilli (3×10^6 bacterial units), and each subsequent tenfold dilution delayed the appearance of the lesion for approximately two weeks. Characteristic swelling developed after periods varying from one hundred to one hundred and thirty-five days after the inoculation of doses containing less than ten bacilli (figure 2). In all cases the swelling of the footpad was progressive. However long the mouse was observed, lesions distant from the site of inoculation were very rarely seen. Localized edema and loss of hair in the inguinal region on the side of the inoculated foot were common late signs. No subcutaneous edema or swellings on appendages other than the inoculated foot were observed, whereas both of these phenomena were noted after inoculation by the intraperitoneal and intravenous routes. Culture of the liver and spleen after footpad inoculation, however, revealed that viable bacilli could be found in these organs for at least five months after inoculation (tables 2 and 7). These bacilli, like the original cultures, grew at 33°C. but not at 37°C.

It is apparent that infection may be established by a very small dose of bacteria inoculated into a site (such as the foot) in which they will readily multiply, and footpad inoculation appeared to be about as sensitive a method as culture for detecting viable bacilli on oleic-acid–albumin–agar medium. Experiments described elsewhere (6) indicate that on this medium approximately 25 per cent of the bacterial units seen when a Tween®-albumin culture is counted in a Petroff-

TABLE 1

RESPONSE OF MICE TO M. ULCERANS STRAIN RS INOCULATED BY VARIOUS ROUTES

Route of Inoculation	Number of Viable Units in Inoculum	Time of Appearance of Lesion	Type of Lesion Produced
		weeks after inoculation	
Footpad	3×10^8	1	Progressive swelling of inoculated footpad
	3×10^4	4–5	Progressive swelling of inoculated footpad
	3×10^2	10	Progressive swelling of inoculated footpad
	3	14–16	Progressive swelling of inoculated footpad
Intranasal	3×10^8	24–30	Generalized skin lesions (feet, tail, scrotum, ears, nose)
	3×10^4		None
Intraperitoneal	6×10^8	10	Generalized subcutaneous edema
	6×10^4	18 and 24	Generalized skin ulceration in 2/6* (feet, tail, scrotum)
	6×10^2	—	None
Intravenous	6×10^8	†4–10; 9–12	Ulcer on tail; generalized skin ulceration (feet, tail, scrotum, ears, nose)
		12	Generalized subcutaneous edema
	6×10^4	10–17; 17–19	Ulcer on tail; generalized skin ulceration (feet, tail, scrotum, ears, nose)
	6×10^2	15–17	Ulcer on tail
Intracerebral	2×10^8	12–16	Generalized skin ulceration (feet, tail, ears) and generalized subcutaneous edema
	2×10^4	16	Generalized subcutaneous edema 4/6
	2×10^2		None

* Numerator = number of mice showing lesions; denominator = number of mice in group.

† First numbers = range of time of appearance of ulcers on tail; second numbers = range of time of appearance of generalized skin ulceration.

Hausser chamber will give rise to colonies. The interval between the first appearance of lesions with successive tenfold dilutions of the culture was, with both strains SF and RS, between ten and fourteen days. If it be assumed that a local lesion becomes apparent after a certain critical concentration of bacilli is reached, it may be inferred that in the footpad there is a tenfold increase in bacterial concentration every ten to fourteen days, i.e., the generation time of the microorganism in the tissues of the mouse foot is about three to four days.

Intravenous inoculation: The effects of the intravenous inoculation of *M. ulcerans* were quite different from the strictly localized effect observed after pad inoculation. Lesions of the tail (at the site of the inoculation puncture) appeared in all mice, at times varying from four to ten weeks after inoculation with the undiluted inoculum to fifteen to seventeen weeks after the inoculation of 6×10^2

FIG. 1. The appearance of the foot of a mouse seven weeks after the inoculation of 10^5 viable bacterial units of *M. ulcerans* strain RT into the pad of the foot. Swelling was first detected three weeks after the inoculation.

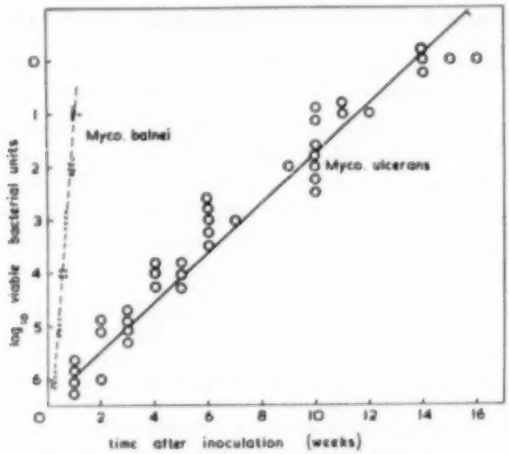

FIG. 2. The relationship between dosage and the times of appearance of the swelling of the foot in mice inoculated in the pad of the hind foot with *M. balnei* strain K_9O or *M. ulcerans* strain R8. Each symbol represents one mouse.

viable bacterial units. The variation within groups inoculated with the same dose was greater than after pad inoculation, which is not surprising, for the dose of bacilli accidentally deposited in the tail during the intravenous inoculation undoubtedly varied considerably. Lesions developed at other sites in all of the mice which received the largest inoculum, in 4 of the 6 which received the dose of 6×10^4 viable bacterial units, and in none of those which received the smallest dose. Swelling and then ulceration occurred commonly on the scrotum, on various

parts of the tail distant from the puncture site, on any or all of the four feet, on the nose, and on the ears—in short on any of the peripheral hairless portions of the body. In addition, some of the mice developed gross edema of the subcutaneous tissues all over the body, similar to that illustrated by MacCallum and associates (1). Generalized peripheral lesions became evident many weeks after the appearance of ulceration of the tail at the puncture site and, with small inocula (6×10^2 bacterial units), none occurred, even after a year.

The results of inoculation by other routes are summarized in table 1, but need not be considered in detail. Local lesions of the inoculated organs never occurred after intranasal or intracerebral inoculations. Nevertheless, in the mice which received the largest dose intranasally, ulceration of the nose, and later of the feet and scrotum, occurred twenty-four to thirty weeks after inoculation. This was presumably due to local injury on inoculation and spread of the bacilli through the blood stream shortly after the intranasal instillation of the large dose of bacteria. In the instances in which lesions did occur after intracerebral and intraperitoneal inoculation, they were of the same type and occurred in the same parts of the body as those just described as a consequence of intravenous inoculation. It is probable that they were due to bacilli distributed during the overflow of the inoculum into the circulation just after inoculation (7).

COMPARISON OF THE REACTION OF MICE TO INFECTION WITH M. ULCERANS AND OTHER MYCOBACTERIA

The relatively rapid development of swelling of the foot after the inoculation of a large dose of *M. ulcerans* into the footpad of the mouse, and the sensitivity of the test, i.e., the appearance of a positive reaction after the inoculation of very small doses of bacilli, indicated that this might be a useful diagnostic test for *M. ulcerans*. It was necessary to determine whether a similar reaction might follow the inoculation of other mycobacteria.

Several species of mycobacteria were grown in Tween®-albumin medium, titrated on oleic-acid-albumin agar, and inoculated intravenously and into the footpad of groups of 4 mice, the undiluted culture and a 1:100 dilution of it being used. The animals were observed weekly, and a record was kept of the degree of swelling of the footpad and of any other lesions. Three months after the inoculation all surviving animals were killed. At autopsy the lungs and spleens were examined. Sometimes cultures were made from them, and in certain cases sections from the internal organs or the inoculated feet were examined for acid-fast bacilli. The results are presented in table 2.

The responses of mice after both intravenous and pad inoculation of *M. ulcerans* differed from those obtained with other species of mycobacteria. The response to intravenous inoculation of mammalian tubercle bacilli varied with the virulence of the culture. Animals inoculated with the undiluted culture of H37Rv died after three to four weeks with typical symptoms of mouse tuberculosis. With BCG, the spleen was somewhat enlarged, and there were a number of small foci of consolidation throughout the lungs. Culture of both organs was positive for mycobacteria. The H37Ra strain caused no changes in the internal organs and could not be cultured from them after three months. *M. tuberculosis*

TABLE 2

THE RESPONSE OF MICE TO INTRAVENOUS AND FOOTPAD INOCULATION WITH SEVERAL DIFFERENT SPECIES OF MYCOBACTERIA

Species of Mycobacterium	Titer of Culture	Response of Mice							
		After Intravenous Inoculation (0.1 Ml. of Undiluted Culture)					Swelling of Foot After Pad Inoculation (0.05 Ml.)		
		Clinical	Autopsy Three Months After Inoculation				Undiluted Culture		10^{-3} Dilution
			Macroscopic Lesions		Culture				
			Spleen	Lungs	Spleen	Lungs	Severity	Duration	
	bacterial units per ml.								
M. tuberculosis var. *hominis* strain H37Rv	42×10^7	Died 3 to 4 weeks	++*	+++	+++*	+++	Slight†	From first week	–
M. tuberculosis var. *hominis* strain H37Ra	8×10^8	None	–	–	–	–	–	–	–
M. tuberculosis var. *muris*	5×10^7	None	±	++	+	++	Slight i 2/6‡ mice	From fourth to eighth weeks	–
M. tuberculosis var. *bovis* strain BCG	8×10^7	None	+	±	+	+	Slight	From first week	–
M. avium	96×10^7	None	+++	–	+++	++	Slight	From first week	Slight transient (3 to 9 weeks)
M. ranae	32×10^7	None	–	–	+	–	–		–
M. phlei	4×10^7	None	–	–	–	–	–		–
M. ulcerans strain RS	5×10^7	Swollen ulcerated feet and tail; generalized subcutaneous edema	–	–	+	+	Progressive, to very severe by 4 weeks	From first week	Progressive from fourth week; severe by 7 weeks
M. balnei strain K_2O	2×10^7	Swollen ulcerated feet, tail, ears, snout	++	–	+	+	Progressive, to very severe by 5 days usually regressed after 6 weeks	From second week	Progressive from fourth day; severe by 9 days, regressed after 6 weeks

* – to +++ arbitrary grades of severity of lesions of liver and spleen and arbitrary grades of density of growth on culture of organs.
† One died of pulmonary tuberculosis; others showed pulmonary lesions.
‡ Numerator = number of mice showing lesions; denominator = number of mice in group.

var. *muris* produced somewhat more severe lesions than BCG. Semi-confluent areas of consolidation occurred in the lungs. *M. avium* caused great enlargement of the spleen and was isolated in high concentration from both the spleen and lungs at autopsy. *M. phlei* and *M. ranae* failed to produce any abnormal signs or symptoms, but a few colonies of *M. ranae* were recovered from the spleen three months after the inoculation.

With *M. ulcerans* there were no lesions of the internal organs, although a few bacilli were cultured from the spleen and lungs, but generalized subcutaneous edema occurred in all animals which had been inoculated with the undiluted culture suspension. These animals also developed ulcers in various peripheral regions, on the tail (probably due to contamination during inoculation), on the feet, nose, ears, and scrotum. Such changes occurred in only one of the 4 animals inoculated with the more dilute suspension, although early ulceration of the tail was observed in all of them.

After pad inoculation also the response of mice to *M. ulcerans* differed greatly from the response to other mycobacteria. Slight swelling, which was not progressive but remained about the same throughout the experiment, was observed in mice inoculated with H37Rv, BCG, *M. tuberculosis* var. *muris*, and *M. avium*. With H37Rv, generalized disease followed the inoculation and one mouse died of pulmonary tuberculosis. With the 1:100 dilution of the culture used as an infecting dose, the results were negative except for the animals which received *M. avium*. In these animals, slight transient swelling of the foot was observed between the third and ninth weeks.

In contrast, with *M. ulcerans*, progressive swelling of the foot, which became extreme, occurred in all cases. With the undiluted culture, this was first observed a week after inoculation and had become very severe by the fourth week. With the smaller infecting dose (1:100 dilution of the culture), the swelling appeared by the fourth week and became severe three weeks later. Ulceration of the foot occurred after the swelling had become extreme, and subcutaneous edema later occurred in the inguinal region on the inoculated side.

These experiments appeared to provide a relatively rapid and specific diagnostic test for *M. ulcerans*. Some time after they were performed, however, Nordén and Linell (2) reported the isolation of an acid-fast organism from cases of cutaneous ulceration in children in Sweden. Like *M. ulcerans* this bacterium failed to grow at 37°C. but grew well at lower temperatures. Dr. Nordén kindly provided cultures of two strains of this microorganism, which has been named *M. balnei* (3, 8), which were used in experiments on mice comparable with those just reported. The results are shown in the lower part of table 2.

M. balnei behaved more like *M. ulcerans* than any other mycobacterium examined, but there were clear differences between them. The progress of symptoms with *M. balnei* was much more rapid than with *M. ulcerans*, and swelling of the foot occurred within two days of the inoculation of a large dose of bacilli and within three to five days of the inoculum of the 1:100 dilution of culture. The development of the lesion was also more rapid, and within seven days of the first appearance of swelling a small pustule appeared on the sole of the inoculated foot

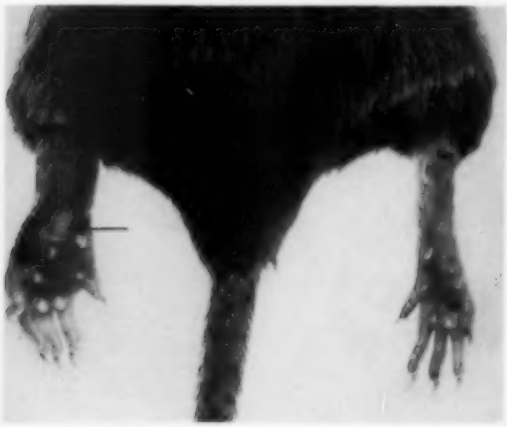

FIG. 3. The appearance of the foot of a mouse ten days after the inoculation of 10^5 viable bacterial units of *M. balnei* strain K_2O into the pad of the foot. Swelling first appeared three days after the inoculation. The arrow indicates the pustule on the sole of the foot.

(figure 3). The pus from this lesion contained numerous very long acid-fast organisms which appeared to consist of chains of very short bacilli.

Pustule formation and such an arrangement of the bacilli have never been observed in infections with *M. ulcerans*. The pustule produced by the *M. balnei* ulcerated within a few days and healing occurred fairly rapidly thereafter. In a few cases generalized lesions occurred after inoculation of *M. balnei* into the footpad. For instance, in one experiment, 4 of 24 mice which were kept under observation for four months after pad inoculation with *M. balnei* strain K_2O developed lesions during the fourth month which contained acid-fast bacilli. The lesions appeared on the tail, scrotum, face, and feet. Six of 11 mice observed for six months developed metastatic lesions.

To test the sensitivity of pad inoculation as a diagnostic test for *M. balnei*, an experiment was performed with serial tenfold dilutions of strain K_2O. The result is recorded in figure 1, which well illustrates the great difference in the rapidity of development of the characteristic swelling of the foot with *M. balnei* and *M. ulcerans*. Minimal inocula of each mycobacterium caused swelling of the inoculated foot in the mouse; but with *M. balnei* this was observed within a week, whereas with *M. ulcerans* it never occurred before the fourth month.

After the intravenous inoculation of *M. balnei*, nodular swellings, which became pustular, developed along the whole length of the tail and, to a varying extent, on the feet, nose, ears, and scrotum (figure 4). The first changes in the tail were noticed seven days after the intravenous inoculation of 10^6 viable units, and became pustular a week later. Involvement of the feet, nose, et cetera was first seen in the third week. Three months after the inoculation a few bacilli could be isolated from the lungs and spleen, but obvious lesions in the lungs were only occasionally seen. The peripheral lesions healed slowly. A few mice inocu-

FIG. 4. The appearance of a mouse thirty-five days after the intravenous inoculation of 10^6 viable bacterial units of *M. balnei* strain K_9O. There were pustular swellings or ulcers on the tail, ears, feet, nose, and scrotum.

lated intravenously with large doses of *M. balnei* died after approximately four weeks. These animals had very severe and widespread peripheral lesions (in tail, all feet, nose, scrotum, et cetera) but at autopsy there were no macroscopic lesions of either spleen or lungs.

Linell and Nordén (3) reported widespread lung consolidation in approximately one-third of the mice inoculated intraperitoneally with a large dose of bacilli (0.25 or 1.0 mg.). In the laboratory of the present writer, *M. balnei* strain K_9O

TABLE 3

THE RESPONSE OF MICE TO INTRAVENOUS AND INTRAPERITONEAL INOCULATION OF LARGE DOSES OF M. BALNEI STRAIN K_2O

Inoculum	Route of Inoculation	
	Intravenous	Intraperitoneal
viable bacterial units		
$10^{7.9}$	19*, 19, 20, 25, K21†	38, 45, S, S, S, S
$10^{6.9}$	45, 46, 52, K89†, S, S	64, 72, S, S, S, S
$10^{5.9}$	66, 80, 100, S, S, S	S, S, S, S, S, S

* Specific death 19, et cetera, days after inoculation

† Killed when moribund

S Survivor at conclusion of experiment (116 days)

failed to produce lung lesions after intravenous, intraperitoneal, or intranasal inoculation. Groups of 6 mice were inoculated intravenously and intraperitoneally with a culture of strain K_2O concentrated ten times and with the undiluted culture fluid and a tenfold dilution of it. The most concentrated inoculum contained $10^{7.9}$ viable bacterial units. The pathologic lesions were much more extensive after intravenous than after intraperitoneal inoculation and their rate of development was proportional to the dosage of bacteria; but in no case did significant lesions of the lungs develop.

Following intravenous inoculation the usual picture was severe pustulation and ulceration of the tail, swelling of the scrotum, lesions on any or all feet, and ulcerating lesions of the ears, which frequently sloughed off. The animals became thin and pale, with distended abdomens, and frequently sneezed and wiped their noses with their paws. Abscesses of the testis and epididymis were always present, and, as Linell and Nordén found, changes attributable to amyloid disease were frequent; but in no case did consolidation of the lungs occur. Small numbers of bacilli were sometimes recovered from the lungs, liver, and spleen, but they did not appear to be associated with specific lesions of these organs (*cf.* table 4).

Intraperitoneal inoculations invariably caused scrotal enlargement, shown by autopsies to be due to the formation of abscesses of the testes and epididymis. With the larger doses, pustular lesions occurred on the feet, tail, nose, and ears of some mice; but they appeared considerably later and were not as extensive and severe as in the groups inoculated intravenously. Abscesses of the testes were the only lesions seen in the mice inoculated intraperitoneally with $10^{5.9}$ viable bacterial units.

The experiment was concluded approximately four months after its inception. The only abnormalities of internal organs were abscesses of the testes or epididymis, which occurred in all animals of both groups, and occasionally abscesses of the brachial or mesenteric lymph nodes. *M. balnei* was recovered in small numbers from the lungs, liver, or spleen of a few animals, but such recoveries were not associated with obvious lesions of these organs.

The failure of *M. balnei* strain K_2O to multiply to any significant extent in the lungs of mice was confirmed by an experiment involving the intranasal inocula-

tion of groups of 6 mice with $10^{6.5}$ and $10^{5.5}$ viable bacterial units. None of the inoculated mice showed any signs or symptoms of infection, and cultures of the lungs nine weeks after inoculation were uniformly negative.

The pathologic picture after the inoculation of *M. balnei* in mice in this laboratory thus differed from that described by Linell and Nordén in that multiplication of the bacilli occurred only in the "low temperature" organs, i.e., the testes and epididymis and the hairless areas of the skin and appendages.

CONTACT INFECTION OF MICE WITH M. BALNEI

Comment was made earlier on the occasional occurrence of cutaneous lesions on the tail and elsewhere in mice inoculated in one foot with *M. balnei*. There are two possible explanations for the development of such lesions: they could result from deposition of bacilli during the "bacteremic showers" which must have occurred during the course of the infection and which were responsible for the occasional isolation of bacilli from the internal organs of mice inoculated in the footpad (tables 2 and 8); or they could be new lesions resulting from contact of the skin with the contaminated environment.

An attempt was made to determine whether contact infection could occur if normal animals were housed with infected mice. Five groups each of 4 normal mice were housed in the same cages as groups of approximately 6 infected mice, some of which had severe ulcerating lesions of the tail, limbs, ears, et cetera, and some severe infections of one foot. The normal mice were examined weekly for ten weeks. Contact infection occurred in one of the 20 mice exposed. This animal, caged with a group of infected mice bearing severe and extensive skin lesions, developed a lesion of the tail four weeks after introduction to the cage, and this progressed in characteristic fashion. The diagnosis was confirmed histologically.

THE PATHOGENESIS OF INFECTIONS OF MICE WITH M. ULCERANS AND M. BALNEI

The distribution of bacilli at various intervals after the infection of mice by the intravenous route, or by pad inoculation, with *M. ulcerans* or *M. balnei* was determined by serial cultural and histologic examinations of the organs of inoculated mice.

The general plan of the experiments was to inoculate groups of mice with known doses of the two strains of mycobacteria, and at various intervals thereafter to kill 2 mice and examine selected organs histologically and culturally.

Material was prepared for culture by grinding weighed amounts in a mortar and pestle with alundum and suspending in 0.5 or 1.0 ml. of 5 per cent bovine albumin. Culture was carried out in several dilutions on oleic-acid-albumin agar, plates being inoculated by the surface drop method (6). Plates inoculated with *M. balnei* were examined after a week's incubation at 33°C., and those inoculated with *M. ulcerans* were examined after five weeks. Portions of the organs examined culturally were fixed, sectioned, and stained by the Ziehl-Neelsen method and counterstained with methylene blue.

M. Balnei Strain K_2O*: Intravenous Inoculation*

Within an hour of the intravenous inoculation of 5×10^5 viable units of *M. balnei* strain K_2O (0.1 ml. of a 1:100 dilution of a well-grown culture), numbers

TABLE 4

THE CONCENTRATIONS OF VIABLE BACILLI IN VARIOUS ORGANS OF MICE INOCULATED INTRAVENOUSLY WITH 5×10^5 VIABLE UNITS OF M. BALNEI STRAIN K_2O

Titers Expressed as Log_{10} Units per Whole Organ

Interval Between Inoculation and Examination	Clinical Signs	Organs Tested					
		Tail	Liver	Spleen	Lungs	Testis-Epididymis	Swollen Foot
1 hour	None	<1.3	5.3	4.1	4.1	—	
2 days	None	<1.3	5.4	4.5	3.7	<1.4	
4 days	None	3.6	5.4	4.4	2.7	3.2	
6 days	None	3.6	5.3	4.7	2.7	4.2	
8 days	Swelling of tail at inoculation site; multiple pustular nodules on tail	—	3.9	2.7	1.4	<1.4	
2 weeks	Swelling of one hind foot (one mouse); enlargement of testis (one mouse)	4.0	3.6	3.6	3.6	6.2	
3 weeks	Multiple pustular nodules on tail; swelling of hindfoot and/or forefoot; swelling on side of face (one mouse)	5.3	3.6	2.2	2.3	<1.4	3.8
4 weeks	Multiple pustular nodules on tail; swelling of both hindfeet and one forefoot; swelling on side of face (one mouse); swelling of scrotum (one mouse)	4.7	3.0	2.4	<1.4	6.9	4.7
6 weeks	Multiple pustular lesions on tail; swelling of both hindfeet and one forefoot; enlargement of one testis	5.2	3.5	2.5	<1.4	7.9	4.2
9 weeks	Lesions of tail healing; swelling of both hindfeet and one forefoot; enlargement of one testis	4.8	2.5	2.2	<1.4	7.9	4.4
13 weeks *A*	Multiple pustular lesions on tail; swelling of all feet; swelling of side of face; enlargement of one testis	—	2.5	3.9	3.8	7.7	5.4
B	Lesions of tail healing; swelling of hindfeet; swelling of testis (one mouse)	—	2.3	3.0	<1.4	—	
20 weeks *A*	Multiple pustular lesions of tail; swelling of hindfeet and one forefoot; swelling of scrotum	6.2	3.9	3.4	4.9	7.4	7.1
B	Lesions of tail almost healed; swelling of hindfeet; enlargement of testis (one mouse)	—	<1.5	<1.4	1.4	6.4	

of bacilli were demonstrable by culture and histologic examination in the liver, spleen, and lungs. The concentration of viable bacilli in these internal organs remained relatively static for a few days and then fell slowly. In the portion of the tail which included the inoculation puncture, on the other hand, the titer rose continuously and rather rapidly. This was associated with obvious inflammatory swelling of the tail, which was first apparent on the eighth day. By the second week there were inflamed pustular nodules along the whole length of the tail, and this condition progressed slowly during the ensuing weeks; in some

animals it commenced to regress by the eighth week. An occasional animal showed a localized swelling on a hindleg or foreleg two weeks after the inoculation. A week later such lesions were almost universal and were shown by culture and section to be due to *M. balnei*. Involvement of the epididymis and testis was not invariable, but occurred rather frequently and was associated with considerable swelling of the affected organ and the presence of large numbers of mycobacteria. Occasionally ulcers occurred on the nose and ears.

After intravenous inoculation, *M. balnei* did not proliferate to any extent in the liver, spleen, or lungs. Any bacilli deposited in an area of relatively hairless skin, or in the testis or epididymis, appeared to multiply and produced pustular swellings similar to those induced in the foot by direct inoculation of the bacilli into the footpad. These swellings slowly regressed, and many of the lesions of the tail had healed five months after inoculation.

The frequent presence of small numbers of viable bacilli in the internal organs for at least twenty weeks after inoculation might be due to long persistence of bacilli deposited at the time of inoculation. The persistence might also indicate slow multiplication in these sites or perhaps the continued deposition there of bacilli which might continually enter the circulation from the peripheral lesions. Cultures grown from twelve colonies which had grown from suspensions of liver, spleen, or lung, three and four months after the inoculation, grew at 33°C. but not at 37°C.

Sections of pustular lesions, whether of the tail, testis, feet, or face, showed abscesses containing very large numbers of greatly elongated acid-fast bacilli, many of which showed pronounced beading.

No microscopic lesions were seen in the sections of the liver, lungs, and spleen. Occasionally isolated acid-fast bacilli were seen within cells, but there was never any accumulation of bacilli suggestive of local multiplication.

M. Balnei Strain K_2O*:Pad Inoculation*

Viable mycobacteria were recovered from the footpad but not from the internal organs immediately after the inoculation of 2×10^5 viable bacterial units of *M. balnei* strain K_2O. The titer in the foot rose steadily to a peak value by the eighth day, at which time early pustulation was apparent. Thereafter the swelling of the foot regressed and the concentration of viable bacilli recovered from it also declined. A good deal of residual swelling was present but no clinical evidence of activity of the infection after the third week. Occasionally samples of the internal organs yielded small numbers of viable mycobacteria. Sections showed only an occasional acid-fast bacillus. Bacilli were not regularly recovered from the regional lymph node, although it was usually considerably enlarged. In a few animals swellings which progressed to pustulation were observed four weeks after inoculation in a foot other than the one which had been inoculated and in the tail. Cultural and histologic examination showed that these were due to *M. balnei*.

Histologic examination of the inoculated foot showed, on the fourth day after inoculation, densely cellular areas containing large numbers of greatly elongated,

TABLE 5

THE CONCENTRATIONS OF VIABLE BACILLI IN VARIOUS ORGANS OF MICE INOCULATED IN THE LEFT HIND FOOT PAD WITH 2×10^5 VIABLE UNITS OF M. BALNEI STRAIN K_3O

Titers Expressed as Log_{10} Units per Whole Organ or Tissue

Interval Between Inoculation and Examination	Clinical Signs	Organs Tested				
		Inoculated Foot	Lymph Node*	Liver	Spleen	Other Tissues
1 hour	None	3.3	<1.4	<1.5	<1.4	
2 days	Inoculated foot slightly enlarged; inguinal lymph node slightly enlarged	4.9	<1.4	<1.5	<1.4	
4 days	Inoculated foot enlarged; inguinal lymph node slightly enlarged	4.7	<1.4	<1.5	<1.4	
6 days	Inoculated foot considerably enlarged; inguinal lymph node slightly enlarged	—	<1.4	3.1	<1.4	
8 days	Inoculated foot greatly enlarged, early pustulation; inguinal lymph node moderately enlarged	5.6	3.0	<1.5	<1.4	
2 weeks	Inoculated foot greatly enlarged, ulcerated; inguinal lymph node moderately enlarged	4.2	<1.4	<1.5	<1.4	
3 weeks	Inoculated foot enlarged, ulcerated; inguinal lymph node enlarged	<1.3	<1.4	<1.5	<1.4	
4 weeks	Inoculated foot enlarged but healing; inguinal lymph node moderately enlarged, swelling of left hind foot and base of tail (one mouse)	2.5	2.1	<1.5	1.5	
6 weeks	Foot lesion regressing; pustular lesion halfway down tail (one mouse)	2.8	<1.4	<1.5	2.0	tail 4.7
9 weeks	Foot lesion regressing	<1.3	1.9	<1.5	<1.4	
13 weeks *A*	Foot lesion regressing; swelling of right hind foot	—	3.5	2.1	2.4	hind-foot 5.2
B	Foot lesion regressing; no secondary lesions	2.4	3.0	<1.5	<1.4	
20 weeks	Foot lesion almost healed, but foot still swollen; no secondary lesions	3.3	<1.4	<1.5	<1.4	

* Inguinal lymph node draining inoculated foot

curved, slightly beaded acid-fast bacilli. In the surrounding tissue there were occasional dense masses of intracellular bacilli. The condition progressed, and the few areas of necrosis visible on the sixth day coalesced to form a large abscess on the eighth day, by which time the bacilli had assumed a very beaded appearance (figure 5). After the abscess had ulcerated, healing commenced, and after the second week only a few beaded and somewhat fragmented acid-fast bacilli could be seen.

Pustular lesions which occurred on sites distant from the foot consisted of small abscesses containing large numbers of elongated curved acid-fast bacilli. These occurred extracellularly throughout the necrotic tissue.

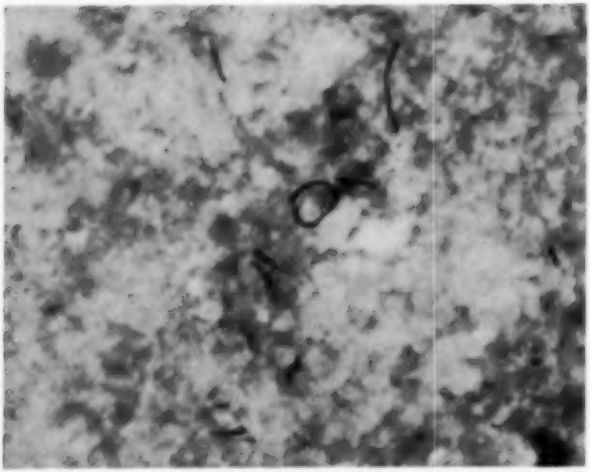

FIG. 5. Elongated beaded bacilli occurring extracellularly in the pustule on the foot of a mouse inoculated six days earlier with 10^5 viable bacterial units of *M. balnei*, strain K_2O. (Ziehl-Neelsen stain. ×1,050)

The Pathogenicity of Colonial Variants of M. Balnei

Both Linell and Nordén (3) and Leach and Fenner (4) recorded the occurrence of variants of *M. balnei* which differed from the original culture in colonial morphology. Linell and Nordén found no differences in the response of mice to the intraperitoneal inoculation of equal doses of their original and the variant strain. The pathogenicities of the original strain and variant isolated in this laboratory, and the cultures kindly sent by Dr. Nordén, were compared by the pad, intravenous, and intraperitoneal inoculation of mice with graded doses of the four strains. Only undiluted cultures and the 10^{-2} dilution were used for intravenous inoculation, and multiple peripheral lesions were produced by both dilutions of all four strains. The results obtained by pad and intraperitoneal inoculation are presented in table 6. No difference was observed between the pathogenic behavior of any of the four strains. Macroscopic consolidation of the lung was not seen. The results showed the much greater sensitivity of pad inoculation compared with intraperitoneal inoculation. Pad inoculations of cultures diluted to 10^{-6} uniformly produced swelling of the foot within three weeks, whereas no lesions were produced within fifteen weeks by doses one hundred times larger inoculated into the peritoneal cavity.

M. Ulcerans Strain RT: Intravenous Inoculation

Within an hour of the intravenous inoculation of 2×10^7 viable bacterial units of *M. ulcerans* strain RT, numerous organisms could be cultivated from the liver, spleen, and lungs, and were histologically demonstrable in these organs. A month after inoculation there was swelling of the tail in the vicinity of the

TABLE 6

THE PATHOGENICITY FOR MICE OF THE ORIGINAL AND COLONIAL VARIANTS OF TWO STRAINS OF M. BALNEI

Strain of *M. Balnei*	Viable Count	Route of Inoculation			
		Pad Inoculation	Intraperitoneal Inoculation		
		10^{-6} Dilution	Undiluted	10^{-2} Dilution	10^{-4} and 10^{-6} Dilutions
	units per ml.				
Strain X					
Original smooth	3×10^8	Swelling and pustulation of inoculated foot by third week	Scrotal and tail lesions by third week; progressive peripheral lesions; no lung consolidation	None	None up to fifteenth week
Rough variant	4×10^7	Swelling and pustulation of inoculated foot by third week	Scrotal and tail lesions by third week; progressive peripheral lesions; no lung consolidation	Scrotal lesions by sixth week in 3/3* mice; no other lesions up to ninth week	None up to fifteenth week
Strain BIV					
Original smooth (BIVX)†	6×10^7	Swelling and pustulation of inoculated foot by third week	Scrotal and tail lesions by third week; progressive peripheral lesions; no lung consolidation	Scrotal lesions by fifth week in 2/3 mice; no other lesions	None up to fifteenth week
Rough variant (BIVT)†	2×10^7	Swelling and pustulation of inoculated foot by third week	Scrotal and tail lesions by third week; progressive peripheral lesions; no lung consolidation	Scrotal and tail lesions by sixth week in 2/3 mice	None up to fifteenth week

* Numerator = number of mice showing lesions; denominator = number of mice in group

† Designations given by Linell and Nordén (3)

TABLE 7

THE CONCENTRATION OF VIABLE BACILLI IN VARIOUS ORGANS OF MICE INOCULATED INTRAVENOUSLY WITH APPROXIMATELY 10^7 VIABLE UNITS OF M. ULCERANS STRAIN RT

Titers Expressed as Log_{10} Units per Whole Organ

Interval Between Inoculation and Examination	Clinical Signs	Organs Tested					
		Tail	Liver	Spleen	Lung	Testis	Swollen Foot
1 hour	None	<2.3	5.9	4.1	4.9	<1.4	
1 week	None	<1.3	4.7	<1.4	4.1	<1.4	
1 month	Swelling of tail at inoculation site	6.9	4.8	4.2	2.6	2.2	
3 months	Considerable swelling of tail; swelling of hindfoot	—	4.2	2.9	3.4	—	4.2
4 months	Swelling of tail, scrotum, hindfoot, one forefoot, snout; gelatinous subcutaneous edema (one mouse)	8.1	4.7	5.0	6.1	—	5.4
5 months	Swelling of tail and hindfeet; swelling of scrotum (one mouse)	6.3	4.1	4.5	<1.4	7.1	5.7

inoculation site, and culture showed that considerable proliferation of bacilli had occurred there. Bacilli were recovered in gradually diminishing numbers from the internal organs. Macroscopic lesions of the feet or testis were accompanied by high titers of bacilli in these sites.

MacCallum and associates (1) described in detail the pathology of experimental lesions in the rat. The histologic features of lesions in the mice were similar. Occasional acid-fast bacilli, occurring intracellularly in small clumps, were seen in the liver, more frequently early in the disease than later. Lesions of the appendages were characterized microscopically by the occurrence of macrophages packed with bacilli. As the lesions progressed, many bacilli grew extracellularly also in long tightly packed "cords." Cultures grown from six colonies obtained from the lungs and liver three and four months after inoculation showed the same temperature requirements as the original culture.

Results quite similar to those described here were also obtained in an experiment with *M. ulcerans* strain RS.

M. Ulcerans Strain RT: Pad Inoculation

An hour after inoculation mycobacteria were demonstrable in the footpad but not elsewhere in mice inoculated in the footpad with 10^7 viable bacterial units of *M. ulcerans* strain RT. Subsequently the titer of bacilli in the foot rose to a maximum three months after the inoculation and then declined somewhat. Although the draining inguinal lymph node was slightly enlarged, secondary lesions in other parts of the body did not occur. After the first month viable bacilli were often recovered in small numbers from the internal organs. On histologic examination, acid-fast bacilli were always seen in the foot but not in the other organs. In the early stages of the infection they were always intracellular, the infected cells being tightly packed with bacilli. After the third month the

TABLE 8

THE CONCENTRATION OF VIABLE BACILLI IN VARIOUS ORGANS OF MICE INOCULATED IN THE LEFT HINDFOOT PAD WITH 10^7 VIABLE UNITS OF M. ULCERANS STRAIN RT

Titers Expressed as Log_{10} Units per Whole Organ

Interval Between Inoculation and Examination	Clinical Signs	Organs Tested				
		Inoculated Foot	Lymph Node*	Liver	Spleen	Lung
1 hour	None	—	<1.4	<1.5	<1.4	—
1 week	Inoculated foot slightly enlarged	5.4	<1.4	<1.5	<1.4	<1.4
1 month	Inoculated foot moderately enlarged; inguinal lymph node moderately enlarged	5.1	3.6	<1.5	1.7	<1.4
3 months	Inoculated foot greatly enlarged; inguinal lymph node moderately enlarged	8.1	2.1	2.0	2.4	2.8
4 months	Inoculated foot moderately enlarged; inguinal lymph node moderately enlarged	6.7	4.1	2.7	2.0	1.7
5 months	Inoculated foot moderately enlarged	5.8	<1.4	3.7	1.7	—

* Inguinal lymph node draining inoculated foot

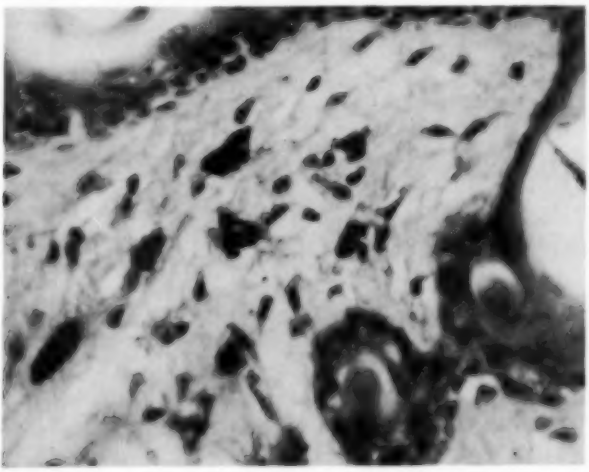

FIG. 6. Macrophages packed with "cords" of acid-fast bacilli in the subcutaneous tissue of the foot of a mouse inoculated in the footpad three months earlier with 10^7 viable bacterial units of *M. ulcerans* strain RT. (Ziehl-Neelsen stain. ×750)

bacterial growth appeared to extend out of the cells, and long tight cords of acid-fast bacilli were found, often apparently originating from the bacilli within a cell (figure 6). Scattered single extracellular acid-fast bacilli were not seen.

Results essentially the same as those just described were also found with *M. ulcerans* strain RS.

TABLE 9

THE GROWTH OF M. BALNEI STRAIN K_2O IN THE LIVER OF DEVELOPING CHICK EMBRYOS INOCULATED INTRAVENOUSLY WHEN 11 DAYS OLD AND SUBSEQUENTLY INCUBATED AT 33°C. OR 37°C.

Temperature of Incubation	Time of Incubation	Liver Suspension			Section of Liver	
		Smear		Viable Count	Average Number of Bacilli per Standard Field	Morphology of Bacilli
		Density of Bacilli	Morphology of Bacilli			
				per ml.		
33° and 37°C.	Inoculum	Numerous	Well dispersed short	$10^{7.6}$	—	—
	1 hour	Scanty	Short, single	$10^{6.8}$	7.7	Scattered, single, short
33°C.	1 day	Scanty	Elongated	$10^{7.9}$	8.6	Elongated
	2 days	Numerous	Elongated	—	>100	Clumps, elongated bacilli, pairs
	3 days	—	—	—	Hundreds	Clumps, elongated bacilli, pairs
	5 days	Very numerous	Single, short	$10^{7.6}$	Hundreds	Clumps, and single shorter bacilli
37°C.	1 day	Scanty	Short	—	8.2	Some elongated, some short
	2 days	Scanty	Short	—	5.7	Some elongated, some short
	3 days	Scanty	Elongated, beaded	$10^{6.3}$	2.9	Some elongated and beaded
	4 days	Very scanty	Elongated, beaded	$10^{5.9}$	1.5	Some elongated and beaded
	5 days	Very scanty	Elongated, beaded	$10^{6.0}$	1.8	Some elongated and beaded
	8 days	Very scanty	Elongated, beaded	$10^{5.0}$	<1	Very elongated, scanty, beaded

THE PATHOGENICITY OF M. ULCERANS AND M. BALNEI FOR CHICK EMBRYOS

The ability to control the temperature of incubation of developing chick embryos over a rather wide range suggested that it might be fruitful to study the response of this host to inoculation with *M. ulcerans* and *M. balnei*.

In preliminary experiments the embryos were inoculated with a concentrated suspension of *M. ulcerans* strain SF by a variety of routes, e.g., yolk sac, amniotic, allantoic, chorioallantoic, and intravenous; and were incubated at 33° and 37°C. The inoculation techniques employed were those described by Beveridge and Burnet (10). While multiplication probably occurred in the yolk sac and on the chorioallantoic membrane, it was soon apparent that more information could be obtained by intravenous inoculation. Accordingly, groups of 18 twelve-day-old chick embryos were inoculated intravenously with 0.1 ml. of undiluted

TABLE 10

THE GROWTH OF M. ULCERANS STRAIN RT IN THE LIVER OF DEVELOPING CHICK EMBRYOS INOCULATED INTRAVENOUSLY WHEN 11 DAYS OLD AND SUBSEQUENTLY INCUBATED AT 33°C. OR 37°C.

Temperature of Incubation	Time of Incubation	Liver Suspension			Section of Liver
		Smear		Viable count	
		Density of Bacilli	Morphology of Bacilli		
				per ml.	
33° and 37°C.	Inoculum	25% single; rest small clumps	Short	$10^{7.7}$	—
	1 hour	Scanty	Single and small clumps, short	$10^{6.4}$	Scattered small clumps of short bacilli
33°C.	1 day	Scanty	Single and small clumps	$10^{6.4}$	Scattered small clumps of short bacilli
	2 days	Scanty	Small clumps	—	Scattered small clumps of elongated bacilli
	3 days	Scanty	Larger clumps	$10^{6.7}$	Scattered small clumps of elongated bacilli
	6 days	Scanty	Larger clumps	$10^{6.0}$	Single and scattered somewhat larger clumps of elongated, well-defined bacilli
	8 days	—	—	$10^{5.7}$	Somewhat larger clumps of elongated, sometimes beaded, bacilli
37°C.	1 day	—	—	—	Scattered small clumps of short bacilli
	2 days	Scanty	Small clumps	—	Scattered small clumps of short bacilli
	3 days	Scanty	Small clumps	$10^{6.1}$	Scattered small clumps of short, degenerated bacilli
	6 days	Very scanty	Small clumps	—	Scattered small clumps of short degenerated bacilli and clumps of acid-fast material
	8 days (1)	—	—	$10^{5.3}$	Very few collections of short bacilli and acid-fast material
	8 days (2)	—	—	$10^{5.9}$	Very few collections of short bacilli and acid-fast material

cultures of *M. ulcerans* strain RT (5×10^7 viable units per ml.) or with *M. balnei* strain K_2O (5×10^7 viable bacterial units per ml.). Eggs were incubated at either 33°C. or 37°C. in incubators which maintained these temperatures to within 0.5°C. throughout the experiment. One hour after the inoculation, and at daily intervals thereafter, one or more embryos from each group were killed and portions of the liver were placed in fixative for subsequent histologic study. Another portion of the liver was thoroughly ground with sand in a mortar and pestle and taken up in 0.5 ml. of 5 per cent bovine serum albumin. Smears of this prep-

aration were stained by the Ziehl-Neelsen method, and the morphology of the bacilli and their numbers were noted; viable counts were made of the same preparation.

The results are summarized in tables 9 and 10.

In eggs incubated at 33°C., *M. balnei* caused a rapidly progressive and fatal infection of the chicken embryo. By the end of the first day the bacilli had become elongated and by the third day they had greatly increased in number. All embryos had died by the fifth day. In another experiment in which 25 embryos were inoculated intravenously with a dose of 6×10^6 viable units of *M. balnei* strain K_2O, 11 died on the third day, 10 on the fourth day, and one on the sixth day; there were 3 apparently healthy survivors on the seventh day after inoculation. The specificity of the deaths was in all cases checked by the examination of smears of the liver. The 3 survivors had probably been successfully inoculated, for a few acid-fast bacilli were seen in smears of their ground-up livers. When the eggs were incubated at 37°C., on the other hand, the microorganism proved innocuous. The bacilli never increased in numbers, the viable count fell off rapidly, and there were no deaths in the group.

The much slower growth rate of *M. ulcerans* rendered the differential response less apparent. In eggs incubated at 37°C. after inoculation, the numbers of viable bacilli declined rapidly; and after eight days bacilli were difficult to find in sections of the liver. The viable count of liver suspensions from eggs incubated at 33°C. remained relatively constant, but the size of the clumps of bacilli seen in sections increased appreciably. This effect was more pronounced in another experiment carried out with a more completely dispersed suspension of bacilli of strain RS. Immediately after inoculation, scattered single bacilli could be seen in sections of the liver and spleen. After eight days' incubation at 37°C., there were scattered groups of two or three short acid-fast rods, whereas the livers and spleens of eggs incubated at 33°C. contained bundles of 8 to 20 elongated bacilli.

The decline in the viable count of organisms in the liver homogenates of embryos incubated at 37°C. is not accountable merely by thermal inactivation of bacilli, for Leach and Fenner (9) found that the viable count of *M. ulcerans* in Tween®-albumin medium remained unchanged after seven days at 37°C.

With both *M. balnei* and *M. ulcerans*, therefore, proliferation in the internal organs of the chick embryo was controlled by the temperature of incubation.

Discussion

Experimental demonstration of the controlling effect of body temperature on pathogenic activity has been reported with viruses (myxoma virus (11)), bacteria (pneumococcus type III (12)), and fungi (*Cryptococcus neoformans* (13)). The evidence just presented indicates that the pathogenic behavior of these two newly described mycobacteria, both in man (in whom they cause cutaneous lesions only) and in experimental animals, is governed by their strict dependence upon a narrow temperature range for growth to occur. Such temperatures occur in the skin and subcutaneous tissues of man and the mouse, and in the latter the lesions are further localized to hairless peripheral areas. Evidence for the failure of these mycobacteria to grow in culture media at temperatures about 35°C. has been presented elsewhere (4).

Serial cultural and histologic examination of the internal organs of mice inoculated intravenously with these mycobacteria indicated that the number of acid-fast bacilli in the internal organs did not increase, but diminished at a very slow rate. The occasional presence of a few viable bacilli in the internal organs of mice originally inoculated in the footpad showed that some of the bacilli detected in the liver and spleen of the mice inoculated intravenously were probably due to uptake by fixed phagocytes during transient bacteremic episodes. The majority, however, must have been deposited at the time of the original inoculation and remained in a viable but nonmultiplying state for several months thereafter. The failure to recover from the internal organs bacilli able to multiply at 37°C. provided additional evidence that organisms recovered from the liver were merely persisting or were transported from a peripheral site and were not multiplying there.

The results obtained after intravenous inoculation of chick embryos, particularly dramatic with the more rapidly proliferating *M. balnei*, excluded the influence of "tissue tropism" as the explanation of the cutaneous location of lesions in man and in the mouse and confirmed the view that the temperature of the immediate environment of the bacilli was of overriding importance.

The long periods which elapsed before peripheral lesions developed after intravenous inoculation suggested that they were initiated by the very small numbers of bacilli deposited in these sites at the time of the inoculation. This interpretation is borne out by the occurrence of such lesions only after the intravenous or intraperitoneal inoculation of very large doses of bacilli.

The results obtained after pad inoculation of mice with *M. balnei* and *M. ulcerans* suggest that this may be a useful diagnostic test in cases of cutaneous ulceration thought to be caused by acid-fast bacteria. Incubators maintained at 33°C. are rarely available in clinical laboratories, whereas a few mice may be more easily obtained. In the writer's experience *M. ulcerans* and *M. balnei* are the only acid-fast bacilli which will produce a progressive swelling of the foot. The slower progression and lack of pustulation serve to distinguish *M. ulcerans* infections. Section of the swollen foot will show myriads of irregularly scattered long-beaded bacilli in the necrotic material in *M. balnei* infections and scattered mononuclear cells packed with acid-fast bacilli, together with some "cords" of extracellular acid-fast bacilli, in the *M. ulcerans* infections.

The occasional occurrence of contact infection among normal mice in contact with mice bearing severe ulcerating lesions due to *M. balnei* was not surprising in view of the susceptibility of these animals to small doses of the organism deposited subcutaneously in the appendages. Contact infection of mice with *M. ulcerans* was not attempted but has been demonstrated in phalangers (14).

Summary

The response of mice to inoculation in the footpad with several species of mycobacteria showed that only *M. balnei* and *M. ulcerans* produced severe progressive swelling of the inoculated foot. The swelling occurred with a few days of *M. balnei* infection and some weeks after *M. ulcerans* infection. Footpad inoculation of mice could be used as a diagnostic test for these bacilli.

No visceral lesions occurred after intranasal, intraperitoneal, or intravenous inoculation of mice with these bacilli; but after a moderate interval in the case of *M. balnei* and a long interval in the case of *M. ulcerans*, ulcerating lesions developed on the hairless peripheral parts of the body and on the scrotum.

Developing chick embryos inoculated intravenously with *M. ulcerans* and *M. balnei* were incubated thereafter at 33° or 37°C. Multiplication of both species occurred in the livers of embryos incubated at 33°C. and, with the more rapidly growing *M. balnei*, the infection was fatal in three to five days. No proliferation occurred in embryos incubated at 37°C.

The pathogenic behavior of these two mycobacteria, like their growth on culture media, is controlled by the local environmental temperature.

Sumario

El Comportamiento Patógeno del Mycobacterium Ulcerans y del Mycobacterium Balnei en el Ratón y en el Embrión de Pollo en Desarrollo

La respuesta de los ratones a la inoculación en la planta del pie con varias especies de micobacterias demostró que únicamente el *M. balnei* y el *M. ulcerans* producían grave edema evolutivo del pie inoculado. El edema se presentó a los pocos días de la infección con *M. balnei* y algunas semanas después de la infección con *M. ulcerans*. La inoculación en la planta de los ratones podría usarse como prueba diagnóstica para dichos bacilos.

No aparecieron lesiones viscerales después de la inoculación intranasal, intraperitoneal o intravenosa de los ratones con esos bacilos, pero después de un intervalo moderado, tratándose del *M. balnei*, y de uno largo, tratándose del *M. ulcerans*, se presentaron lesiones ulceradas en las partes periféricas lampiñas del cuerpo y en el escroto.

Los embriones de pollo en desarrollo, inoculados endovenosamente con *M. ulcerans* y *M. balnei*, fueron incubados después a 33° o 37° C. Observóse multiplicación de ambas especies en el hígado de los embriones incubados a 33° C., y con el *M. balnei* de desarrollo más rápido, la infección resultó letal en tres a cinco días. No hubo proliferación en los embriones incubados a 37° C.

El comportamiento patógeno de estas dos micobacterias, lo mismo que su desarrollo en medios de cultivo, es regido por la temperatura ambiente local.

Resume

Comportement pathogène du Mycobacterium ulcerans et du Mycobacterium balnei chez la souris et l'embryon de poulet en cours de développement

La réponse de la souris à l'inoculation dans la plante de la patte de diverses espèces de mycobactéries montre que seuls le *M. balnei* et la *M. ulcerans* produisent la tuméfaction du pied inoculé. Cette tuméfaction apparaissait en quelques jours après l'inoculation avec le *M. balnei* et en quelques semaines après l'inoculation avec le *M. ulcerans*. L'inoculation de la patte de la souris pourrait servir de test diagnostique pour ces bacilles.

Il ne s'est pas produit de lésions viscérales chez les souris à la suite de l'inoculation intranasale, intrapéritonéale ou intraveineuse de ces bacilles; mais après un intervalle modéré, dans le cas du *M. balnei*, et prolongé, dans le cas du *M. ulcerans*, des lésions ulcéreuses se sont développées sur les parties glabres du corps et sur le scrotum.

Les embryons de poulet en cours de développement inoculés par voie veineuse avec le *M. ulcerans* et le *M. balnei* furent soumis à l'incubation à 33 ou à 37°C. La reproduction de ces deux espèces s'est produite dans le foie des embryons incubés à 33°C. et, avec le *M. balnei* qui se développe plus rapidement, l'issue de l'infection fut fatale en trois à cinq jours. Aucune prolifération n'a eu lieu chez les embryons incubés à 37°C.

Le comportement pathogène de ces deux mycobactéries ainsi que leur croissance sur les milieux de culture, est influencé par la température locale ambiante.

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CORDING AND NEUTRAL RED REACTION OF TWO AVIRULENT STRAINS OF MYCOBACTERIUM[1,2]

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INTRODUCTION

Among the *in vitro* tests for virulence of acid-fast organisms which have been proposed, the neutral red reaction and cording, i.e., the parallel orientation of cells to form cords, are considered by many to be the most promising (1–6). Wayne has recently developed a method whereby cording and the neutral red reaction may be observed simultaneously on intact colonies (7). Using this method, Krasnow, Wayne, and Salkin (8) have concluded that a strain which is negative by either of these two criteria is not virulent, while a strain positive by both criteria is virulent or "potentially virulent."

One discrepancy is apparent in the literature. Richmond and Cummings (3) have reported that *Mycobacterium spp. "radish bacillus"* is neutral red positive and corded, while Krasnow and associates (8) find that the same saprophyte, although neutral red positive, is not corded.

The present report concerns a number of factors which may seriously affect both the neutral red reaction and the cording of a strain. It is concluded that extreme caution is advisable in the interpretation of results.

MATERIAL AND METHODS

Two strains of mycobacterium were investigated: *M. spp. "radish bacillus"*[3] and *M. tuberculosis* H37Ra. When strains are serially transferred, using mass inocula, they tend to undergo changes, and for this reason strains of H37Ra were obtained from two different laboratories and labeled H37Ra,[4] and H37Ra$_{st}$.[5]

Liquid cultures were grown in a modified Proskauer and Beck medium (9) containing 0.5 per cent bovine albumin fraction V[6] and 0.02 per cent Tween® 80.[7]

The solid media used were Löwenstein-Jensen medium,[8] modified Proskauer and Beck medium containing 1.5 per cent agar and 0.5 per cent bovine albumin fraction V, and Difco's Dubos Broth Base, also containing 1.5 per cent agar and 0.5 per cent bovine albumin fraction

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[1] From the Investigative Medicine Service, Veterans Administration Hospital, Madison, Wisconsin.

[2] This study was aided by a grant from the Medical Research Section of the American Trudeau Society, the medical section of the National Tuberculosis Association.

[3] Kindly supplied by Dr. Lawrence G. Wayne, Veterans Administration Hospital, San Fernando, California.

[4] Kindly supplied by Dr. Guy P. Youmans, Northwestern University Medical School, Chicago, Illinois.

[5] Kindly supplied by Dr. William Steenken, Jr., Trudeau Laboratory, Trudeau, New York.

[6] Pentex, Inc., Kankakee, Illinois.

[7] Hilltop Laboratories, Cincinnati, Ohio.

[8] Kindly supplied by Dr. F. Kiechle of the Veterans Administration Hospital, Madison, Wisconsin.

V. Since results obtained on the two semisynthetic media were identical in all experiments, these two media will be referred to only as "semisynthetic medium."

All dilutions were made in sterile 0.1 per cent bovine albumin fraction V.

Strips of molecular filter membrane[3] were cut to approximately 0.75 cm. by 1.5 cm., placed between two pieces of filter paper in a Petri dish, and autoclaved for ten minutes at 15 pounds pressure. After autoclaving, the membranes were dried under vacuum in a desiccator. The membranes were then placed on the medium by means of sterile forceps and inoculated with a loop. Before the medium was poured into the Petri dishes, two pieces of 11 cm. filter paper (either Whatman No. 1 or No. 40) were pressed into the top of the Petri dish. After the membrane strips had been inoculated, between 2 and 3 ml. of sterile water were pipetted onto the filter paper to assure a moist atmosphere for growth. The Petri dishes were placed in plastic bags and incubated at 37.5°C.

The neutral red tests were performed by the method of Wayne (7).

RESULTS

"Radish bacillus": Neutral red reaction: M. spp. "radish bacillus" was strongly neutral red positive on all media tested. These results are in agreement with those previous reported (3, 8).

"Radish bacillus": Cording reaction: On Löwenstein-Jensen medium this strain did not show cord formation, i.e., the cells grew in a totally random fashion. On semisynthetic medium, however, the strain did grow in "cords." This effect is shown in figures 1 and 2, which represent seven-day-old neutral red positive colonies of this organism grown on the molecular filter membrane strips. A striking difference was apparent in both gross colony morphology and the arrangement of the cells between colonies grown on the two types of media. Thus the discrepancy between the results of Krasnow and associates (8) and Richmond and Cummings (3) are explained.

H37Ra: Neutral red reaction: The neutral red reaction of *M. tuberculosis* H37Ra was strongly influenced by inoculum size, age of the colonies, and medium.

Essentially identical results were obtained with $H37Ra_{ss}$ and with $H37Ra_{y}$. On semisynthetic medium, young colonies of H37Ra were neutral red positive; as the colonies aged, they became progressively less neutral red positive until they were finally completely neutral red negative. Since it is impossible to reproduce the inoculum size precisely, a certain variation in the time at which the colonies turned neutral red negative was unavoidable. In general, it can be stated that, when a large inoculum (a loop of an undiluted turbid culture) was placed on the membrane strip, the colonies were neutral red negative after approximately ten to twelve days of incubation. When a small inoculum (a loop of a 1:100 dilution of a turbid culture per membrane strip) was employed, some colonies remained neutral red positive as long as fifteen, and occasionally for seventeen, days of incubation.

Essentially similar results were obtained on Löwenstein-Jensen medium. In this instance, however, the neutral red reaction tended to give positive results after somewhat longer periods of incubation. After approximately two weeks of incubation, the colonies were very dense and the neutral red negative colonies were deep orange in color. It was sometimes very difficult to distinguish these

[3] Millipore Filter Corp., Watertown, Massachusetts.

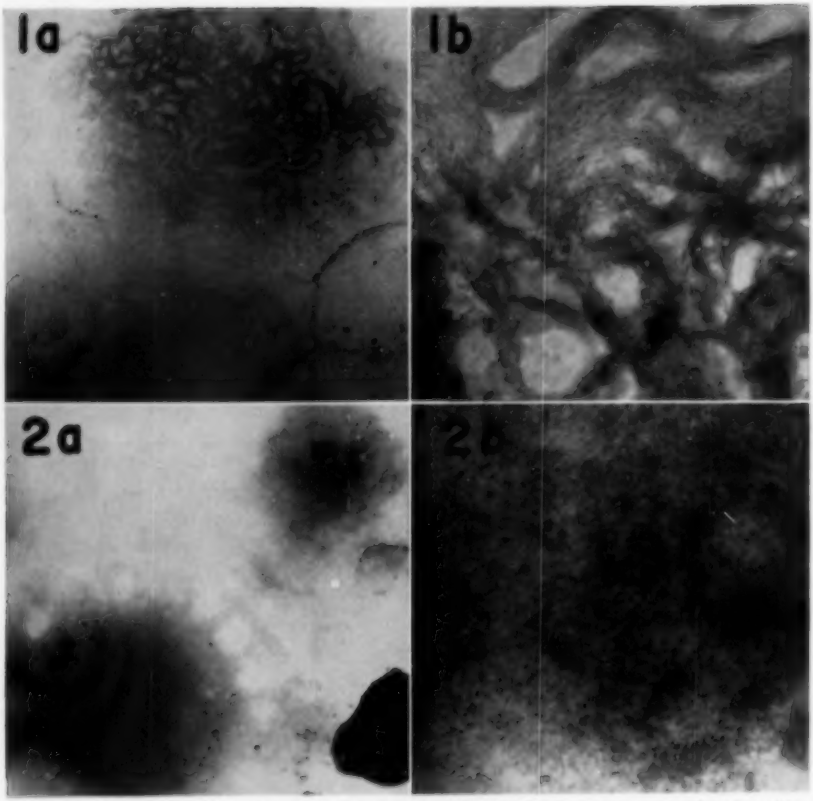

FIG. 1. Seven-day-old colony of *M. spp. "radish bacillus"* grown on molecular filter membrane strips on semisynthetic medium; neutral red positive and corded.

a. ×100.

b. ×610.

FIG. 2. Seven-day-old colony of *M. spp. "radish bacillus"* grown on molecular filter membrane strips on Löwenstein-Jensen medium; neutral red positive and noncorded.

a. ×100.

b. ×610.

colonies from truly neutral red positive colonies, and the scoring necessarily became somewhat arbitrary. Before growth became too dense, neutral red negative colonies, which had the same color as the background, would disappear when the preparation was made transparent with immersion oil. With very dense growth, this test could not be used.

H37Ra: Cording reaction: Colony morphology and "cording" were found to be strongly influenced by the medium employed, and a significant difference was observed between the substrains $H37Ra_y$ and $H37Ra_{st}$.

Both substrains of H37Ra showed a serpentine pattern of growth on semi-

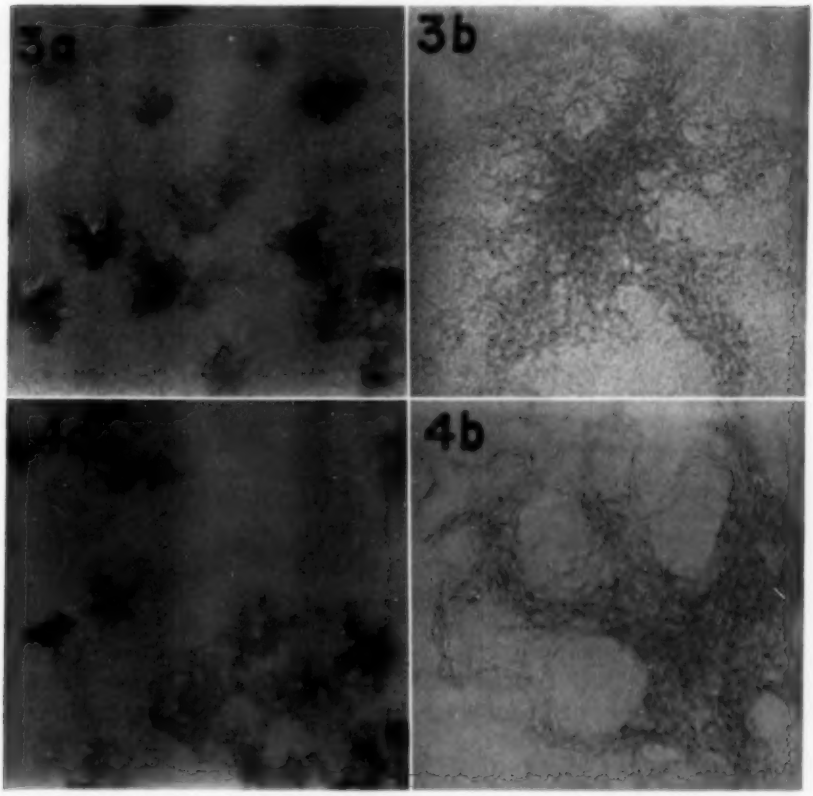

FIG. 3. Ten-day-old colonies of $H37Ra_n$ grown on molecular filter membrane strips on semisynthetic medium; neutral red positive and serpentine.

a. ×100

b. ×610

FIG. 4. Ten-day-old colonies of $H37Ra_y$ grown on molecular filter membrane strips on semisynthetic medium; neutral red positive and serpentine.

a. ×100.

b. ×610.

synthetic medium (figures 3*a* and 4*a*). Examination of the preparation with oil immersion revealed, however, that the cells of $H37Ra_n$ (figure 3*b*) showed little or no parallel orientation at any age, while those of $H37Ra_y$ (figure 4*b*) had a definite tendency to parallel orientation. This parallel orientation was most marked with young preparations but was evident in all neutral red positive preparations. Neutral red negative preparations cannot be examined under oil immersion since the cells are the same color as the background and become invisible when the preparation is made transparent with immersion oil.

On Löwenstein-Jensen medium, both substrains gave identical results. The

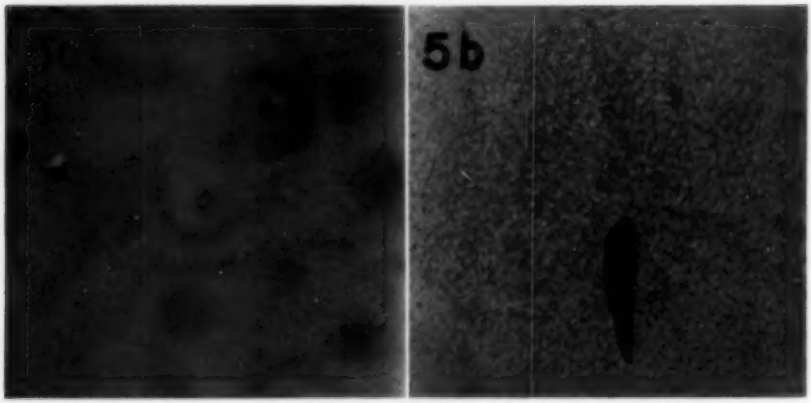

FIG. 5. Ten-day-old colonies of H37Ra$_v$ grown on molecular filter membrane strips on Löwenstein-Jensen medium; neutral red positive and nonserpentine.
a. ×100.
b. ×610.

colonies were round and smooth and showed no serpentine growth pattern whatsoever (figure 5). The cells of even very young preparations (six days of incubation) were arranged completely at random.

Changes in the neutral red and cording reactions associated with the serial transfer of a culture: One culture of H37Ra$_v$ was serially transferred in liquid Proskauer-Beck-Tween®-albumin medium for approximately eighteen months. On solid semisynthetic medium, this culture showed some slight differences from H37Ra$_v$. The colonies were slightly flatter and the neutral red reaction was weaker. When large inocula were used, the colonies became neutral red negative after as few as seven to nine days of incubation. On Löwenstein-Jensen medium, this same culture gave a strong neutral red reaction; even large inocula often remained neutral red positive until the termination of the experiment after three weeks of incubation.

DISCUSSION

The writers believe that an *in vitro* virulence test is of importance primarily in the classification of atypical organisms isolated from patients. With this consideration in mind, the results obtained with *M. spp.* "*radish bacillus*" might be thought of as having little practical significance. However, an organism like H37Ra might well be obtained as an atypical isolate from a patient. By varying the medium, inoculum size, and incubation time, this strain can be made to appear in any one of the four possible forms: neutral red positive and corded, neutral red negative and serpentine, neutral red positive and not "corded," or neutral red negative and not serpentine. In addition to the above considerations, it has been reported that the "cording" reaction may be seriously affected by the presence of bacterial contaminants on the culture medium (10, 11). If the

neutral red reaction or "cording," alone or in combination with each other, are to have any significance in the classification of atypical isolates of acid-fast organisms from patients, a severe standardization of procedure is essential. The question then arises whether the most severe or the least severe test should be applied to these isolates. For example, should suspicion be aroused by any strain which is neutral red positive and corded after a short period of incubation on semisynthetic medium? Conversely, should only those organisms be considered as potentially virulent which are capable of giving a positive neutral red reaction and "cording" after prolonged incubation of a large inoculum on Löwenstein-Jensen medium?

SUMMARY

The neutral red reaction and cording of *Mycobacterium tuberculosis* H37Ra and *M. spp. "radish bacillus,"* grown on molecular filter membranes placed on Löwenstein-Jensen and semisynthetic media, were studied. It was found that inoculum size, incubation time, and type of medium significantly affected these reactions.

SUMARIO

El Encordonamiento y la Reacción del Rojo Neutro de Dos Cepas Avirulentas del Mycobacterium

Se estuaiaron la reacción al rojo neutro y el encordonamiento del *Mycobacterium tuberculosis* H37Ra y del *M. spp. "bacilo del rábano,"* cultivados en membranas-filtros moleculares colocadas en medio de Löwenstein-Jensen y semisintéticos. Observóse que el tamaño del inóculo, el tiempo de incubación y la clase de medio afectaban en forma importante dichas reacciones.

RESUME

La formation de cordons et la réaction au rouge neutre de deux souches avirulentes de Mycobacteries

La réaction au rouge neutre et la formation de cordons du *Mycobacterium tuberculosis* H37Ra et du *M. spp. "bacille radis"*, cultivés sur des membranes moléculaires filtrantes placées sur des milieux synthétiques Löwenstein-Jensen et semi-synthétiques, ont été étudiées. On a observé que le volume de l'inoculum, le temps d'incubation et le type du milieu influençaient ces réactions de façon significative.

Acknowledgment

The writers are indebted to Mr. Forest Fischer, Medical Illustrator of the Veterans Administration Hospital, Madison, Wisconsin, for the skillful photographic work connected with these experiments.

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A CORRELATED BRONCHOGRAPHIC AND HISTOPATHOLOGIC STUDY OF BRONCHIAL DISEASE IN 216 TUBERCULOUS PATIENTS[1,2]

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INTRODUCTION

The clinical value of obtaining bronchograms is to determine whether or not bronchiectasis is present (1). In the pre-surgical evaluation of patients, considerable information can be garnered from the use of bronchograms. This is particularly true in the present day when the segmental resections, subsegmental and wedge resections, are so commonly used. As disease clears with antimicrobial therapy, the exact extent of a resection can be planned with considerable accuracy before a patient is subjected to surgery. The extent of the bronchiectasis or the absence of bronchiectasis may be a very strong factor in determining whether a patient should have a lobectomy, a segmental, or a subsegmental resection.

MATERIALS AND METHODS

Two hundred and sixteen consecutive patients are included in this study. Before bronchoscopy, the patients took nothing by mouth after the previous midnight, 0.1 gm. of sodium pentobarbitol at bedtime and again one hour before the scheduled time of bronchoscopy, 0.010 gm. of morphine, and 0.4 mg. of atropine twenty minutes prior to bronchoscopy. Local anesthesia was used. The mouth and posterior nasopharynx were sprayed with 0.5 per cent solution of Pontocaine® and then 2 to 4 ml. of 2 per cent Pontocaine® were instilled into the trachea under direct visualization. There was no evidence of Pontocaine® sensitivity in this group of patients.

All of the patients were bronchoscoped and at the termination of bronchoscopy a catheter was inserted into the trachea and the patients were immediately taken from the bronchoscopy room to the Department of Radiology. Five to ten milliliters of Iodochlorol® were instilled in each lung under fluoroscopic visualization to adequately outline the entire bronchial tree. There were 191 bilateral bronchograms, 36 bronchograms of the right lung, 29 bronchograms of the left lung; and it was necessary to repeat 40 bronchograms.

There were only 14 patients in the group in which endobronchitis, grade I, was found in the form of edema, granulation, or reddened mucosa. Fourteen bronchograms were done immediately following bronchoscopies on these 14 patients, with evidence of good filling and no evidence of postbronchographic spread or complications.

In order to collect the material to be presented, it was necessary to review the available roentgenograms of all of these patients from the beginning of the disease through its entire hospital course, including their discharge from the hospital. The surgical pathologic specimens were examined by Dr. Ingrid Stergus, pathologist at Battey State Hospital, and the correlation work was then carried out. There were 84 patients in the series who had had pulmonary resections.

[1] From the Battey State Hospital, Rome, Georgia.

[2] Presented, in part, before the Medical Session, as part of *Section 4A*, at the annual meeting of the National Tuberculosis Association, Milwaukee, Wisconsin, May 24, 1955.

TABLE 1

CORRELATION BETWEEN BRONCHOGRAMS AND PATHOLOGIC CHANGES

		Diagnostic Accuracy	
		Per Cent	
Bronchiectasis			
(+) Bronchography (+) Pathologic specimen	42	76	100
(−) Bronchography (+) Pathologic specimen	13	24	
No bronchiectasis			
(−) Bronchography (−) Pathologic specimen	20	80	100
(+) Bronchography (−) Pathologic specimen	5	20	
Stenosis, shown by bronchography and bronchoscopy	2	100	
Fistulas, shown by bronchography and proved by resection	2	100	
Total	84		

OBSERVATIONS

Eighty-four patients in this series had pulmonary resections during their active treatment for pulmonary tuberculosis. These included segmental resections, lobectomies, pneumonectomies, and combinations of these procedures.

Correlations Between Bronchograms and Pathologic Changes (Table 1)

The bronchograms and the pathologic interpretations were reviewed and correlated in the following manner:

Bronchiectasis: The pathologic diagnosis of bronchiectasis was made on 55 of these 84 specimens. Forty-two (76 per cent) of these bronchiectatic lesions were correctly diagnosed by bronchography. An additional 13 (24 per cent) were diagnosed pathologically and were not detected by bronchography.

No bronchiectasis found: Twenty-five of the specimens did not reveal bronchiectasis pathologically. The absence of involvement was demonstrated in 20 (80 per cent) of the specimens by bronchogram and pathologic study. Five specimens without bronchiectasis had been interpreted bronchographically as showing bronchiectasis.

Bronchial stenosis: There were 2 patients who showed stenosis of their main stem bronchus by bronchoscopy; this was also well demonstrated by bronchography and pathologic study.

Fistulas: There were two additional bronchopleural fistulas proved by bronchography and by resection.

Criteria for Diagnosis of Tuberculous Bronchiectasis (Table 2)

Bronchiectasis may develop as the result of dilatation behind a point of obstruction by destruction of a bronchial wall from an inflammatory process, or by the contraction and healing of parenchymal disease causing traction-dilatation of a previously weakened bronchus. A clinician treating a patient with tuberculosis, who discovers bronchiectasis by bronchography in an area where

TABLE 2

CRITERIA FOR DIAGNOSIS OF TUBERCULOUS BRONCHIECTASIS

	Number
Bronchographic evidence of bronchiectasis	21
Gross pathologic evidence of bronchiectasis	21
Histopathologic tuberculosis in an area of gross pathologic bronchiectasis	21
Culture positive for *Mycobacterium tuberculosis* from a noncavitary draining bronchus meeting the above three criteria	8

there has been roentgenographic evidence of tuberculosis, often assumes that the bronchiectasis is tuberculous. The writers believe that a diagnosis of tuberculous bronchiectasis may be made only if there is bronchographic evidence of bronchiectasis; if there is gross pathologic evidence of bronchiectasis in the same area; and if there is histopathologic tuberculosis in an area of gross and bronchographic bronchiectasis. Additional confirmation may be had by obtaining a culture positive for *Mycobacterium tuberculosis* from a noncavitary draining bronchus which meets the previous criteria.

In this series of 84 resected specimens, 55 showed gross evidence of bronchiectasis. Forty-two of these same pathologic specimens had revealed bronchiectasis on a bronchogram. Thirty-one of the surgical specimens revealed histopathologic evidence of tuberculous endobronchitis, but not all in association with a dilated bronchus. There were 10 cases in which cultures positive for tubercle bacilli were obtained from bronchiectatic bronchi revealing tuberculous endobronchitis, but two of the positive cultures came from bronchi-draining open cavities.

Of the 55 specimens that revealed bronchiectasis grossly, there were 21 (40 per cent) that met the criteria of tuberculous bronchiectasis. These 21 specimens all had bronchiectasis on bronchogram, gross pathologic evidence of bronchiectasis, and pathologic evidence of tuberculous endobronchitis in a grossly dilated bronchus. Of these 21 specimens, 8 were from patients from whom a culture positive for *Mycobacterium tuberculosis* was obtained from the same grossly dilated bronchus that revealed histopathologic evidence of tuberculous endobronchitis without draining an open cavity.

Incidence of Bronchiectasis with Various Types of Parenchymal Lesion (Table 3)

The incidence of bronchiectasis following various types of parenchymal lesion was studied. The roentgenograms of the patients with these lesions were reviewed and the tuberculous disease was classified as pneumonic, cavitary, "honeycombing," fibrocaseous, fibrocalcific, exudative, or mixed. In this group of patients, more than one type of pathology could have been found in an individual patient. For example, one patient might have had a cavitary lesion in the right upper lobe and a pneumonic lesion in the left lower lobe. In this way both of these lesions would have been included in this study. The bronchograms were all studied on an anatomic, segmental basis (2). If a patient's original roentgenogram showed a cavitary lesion located in the apical, posterior segment of the left upper lobe, the subsequent bronchogram was interpreted to show whether or

TABLE 3

CORRELATION BETWEEN PARENCHYMAL LESIONS AND RESULTING BRONCHIECTASIS

Type of Lesion	Number of Lesions Studied	Number Positive for *M. tuberculosis*	Per Cent Positive for *M. tuberculosis*
Pneumonic	31	18	58
Cavitary	75	42	56
Honeycombing	31	17	55
Fibrocaseous	54	19	35
Fibrocalcific	15	5	33
Exudative	111	31	28
Mixed	60	13	22

TABLE 4

POST-BRONCHOGRAPHIC SPREAD OF DISEASE

Patients	216
Tuberculous sputum	147
Bronchograms	256
Spread of disease	3
Incidence	
Per bronchogram	1.2%
Per patient	1.4%

not the segmental bronchi leading into the area where the cavity was originally located did or did not show evidence of bronchiectasis. Similarly, the bronchi leading into the area where the pneumonic lesion had originally been located were studied to see whether or not there was evidence of bronchiectasis on the bronchogram.

In the 31 areas where pneumonic tuberculosis was originally present, the segmental bronchi revealed bronchiectasis in 18 instances (58 per cent). Forty-two (56 per cent) of the bronchi leading into the areas where cavitary disease had once been clearly defined revealed bronchiectasis. Seventeen (55 per cent) of the bronchi leading into areas of honeycombing revealed bronchiectasis. Of the 54 fibrocaseous lesions studied, the bronchi on 19 (35 per cent) of the bronchograms revealed bronchiectasis. Five (33 per cent) of the bronchograms of the 15 fibrocalcific lesions revealed bronchiectasis. Thirty-one (28 per cent) of the 111 exudative lesions revealed bronchiectasis. Thirteen (22 per cent) of the 60 mixed lesions revealed bronchiectasis.

Post-Bronchographic Spread of Disease

The incidence of post-bronchographic spread of disease was determined following the double procedure of bronchoscopy and bronchography.

In table 4, it may be seen that there were 256 bronchograms performed on 216 patients, of whom 147 had tubercle bacilli in the sputum at the time of the bronchoscopy and bronchography. Three patients developed a post-bronchographic spread of disease, and all had tuberculous sputum and cavitary disease. The incidence of spread of disease per bronchogram was 1.2 per cent. The incidence of spread of disease per patient was 1.4 per cent.

TABLE 5

ALLERGIC REACTIONS FOLLOWING BRONCHOGRAPHY

	Number of Patients
Elevation of temperature	21
Fever, edema, generalized aching	2
Fever, no edema, generalized aching	2
Allergic conjunctivitis	1
Total	26
Incidence per procedure	10%

Allergic Reactions Following Bronchography

The incidence of allergic reactions following bronchography was investigated and it was found that there were four types of allergic reactions which had occurred (table 5).

The most common allergic reaction was that of temperature elevation alone. All of these patients had had a normal temperature for some time prior to bronchoscopy and bronchography. There were 21 patients in the series who had a slight temperature elevation of 99 to 99.4°F. that lasted approximately three days.

There were 2 patients who exhibited fever, which rose to 102°F. within a few hours following bronchography, edema of the face and eyelids which lasted forty-eight hours, and generalized aching. One of these patients also had edema of the throat and swelling of the posterior nasopharynx, with difficulty in swallowing. In both, the syndromes subsided completely in forty-eight hours.

There were 2 other patients with fever up to 102°F., associated with generalized aching, but with no visible edema. One of these patients continued to run a fever of 102°F. for ten days with gradual subsidence thereafter. The other patient's fever lasted seventy-two hours.

The fourth group was represented by one patient having an allergic conjunctivitis, associated with headache and dizziness, which lasted fifteen days.

Twenty-one of the patients who had allergic reactions did not require specific therapy. Five of the 26 patients had reactions that required antihistaminic therapy. There were no lasting sequelae following any of the reactions.

Oil Retention Following Bronchography (Table 6)

An attempt was made to determine whether there was enough oil retention to seriously interfere with subsequent roentgenographic interpretations.

Fifteen patients left the hospital prior to having a post-bronchographic roentgenogram. The remaining patients received post-bronchographic roentgenographic follow-up. Forty-four (17 per cent) of this group of patients showed no evidence of oil in the post-bronchographic roentgenogram. One hundred and fifty-one patients (59 per cent) showed a slight amount of oil retention. Twenty-nine patients (11 per cent) showed a moderate amount of oil retention. Seventeen patients (7 per cent) had marked oil retention. There were four occasions in this series of 256 bronchograms in which the retention of oil seriously interfered with ability to follow the course of the pathology roentgenographically.

TABLE 6

STUDY OF OIL RETENTION

	Number	Per Cent
No follow-up roentgenogram	15	6
None	44	17
Slight	151	59
Moderate	29	11
Marked	17	7
Total	256	100

TABLE 7

EXTENT OF BRONCHIECTASIS IN A GROUP OF PATIENTS WHO HAD NOT HAD RESECTIONS

3 lobes involved	12
(*a*) unilateral	8
(*b*) bilateral	4
2 lobes involved	5
1 lobe	9
1 or 2 segments only	10
Total	36

Extent of Bronchiectasis in Patients Who Had No Pulmonary Resections

There were 46 patients in this group who had roentgenographic evidence of bronchiectasis and were discharged from the hospital for various reasons, without surgical excision. It was possible to obtain follow-up information on 36 of the 46 patients. Five of these patients, however, had had surgical collapse in the form of thoracoplasty.

The extent of the bronchiectasis in this group of patients may be seen in table 7. Twelve patients had bronchiectasis in at least three lobes with all segments involved. Five had two lobes completely involved with bronchiectasis and 9 had one lobe completely involved. Ten patients had one or two segments alone involved by bronchiectasis. Thirteen of the patients exhibited saccular bronchiectasis; in only 3 of them was cylindrical bronchiectasis noted, while 20 had both cylindrical and saccular bronchiectasis on roentgenograms.

The patients were followed for an average of nineteen months per patient after discharge, and the follow-up brought forth the following information:

No patient had exhibited profuse or foul expectoration.

One patient had had one slight hemoptysis.

The roentgenograms of 32 patients (89 per cent) remained stationary after discharge.

Four patients (11 per cent) had roentgenograms showing improvement following discharge. There was no evidence of worsening.

Two patients had died from nontuberculous causes.

Three patients had bacteriologic relapse with reappearance of tubercle bacilli in the sputum.

One of these patients who had had bacteriologic relapse had extensive bronchiectasis of the right upper and right middle lobes. Another patient had mild saccular and cylindrical bronchiectasis of two basilar segmental bronchi of the left lower lobe, and the third had cylindrical bronchiectasis of one apical segment. These 3 patients who relapsed bacteriologically had received less than six months of streptomycin therapy alone prior to discharge. In no case was the bacteriologic relapse accompanied by evidence of roentgenographic activity of the pulmonary tuberculosis.

DISCUSSION

The importance of adequate, local anesthesia prior to bronchoscopy cannot be overemphasized. This is probably the most common cause of failure to obtain an adequate bronchogram. It is also important that the filling of the bronchial tree be observed under fluoroscopic visualization and that the patient be positioned to obtain this filling. The patients were encouraged to cough and expectorate oil immediately following bronchography, and then were instructed to carry out dependent drainage three times a day for a period of one week following the bronchography.

The interpretations of bronchograms are generally quite accurate. In the present study, it was found that there had been some tendency both to "under-read" and to "over-read" some of the bronchograms. In cases in which there was an opportunity to correlate the bronchographic interpretation with the surgical pathologic specimens, it was found that 24 per cent of the bronchograms had been "under-read" and 20 per cent had been "over-read." Buckles and associates (3) found that they "over-read" bronchograms in approximately the same proportions. Among their patients eventually subjected to resection, bronchiectasis was diagnosed roentgenographically in 73.2 per cent but was confirmed by postoperative microscopic study in only 53 per cent, a difference of 20 per cent. They made no mention of "under-reading" their bronchograms but, in the present study, it was found that this also occurred and in about the same number of instances as "over-reading." P. Chadourne and associates (4) have suggested that more accurate bronchographic information may be obtained by combining it with tomography, doing this in a single examination. They use a mixture of lipiodol, sulfamide, or talc.

In the present study criteria have been set forth defining what the writers designate as tuberculous bronchiectasis. Buckles and associates (3) differentiated sharply between tuberculous bronchitis with bronchiectasis and pure tuberculous bronchiectasis. They applied the former term to dilatation of bronchi accompanying tuberculous bronchitis. They used the designation tuberculous bronchiectasis only in cases in which there were locally destructive effects of tuberculosis on bronchial walls. Only 2 per cent of their surgical specimens revealed tuberculous bronchiectasis; 26 per cent of their surgical specimens revealed bronchiectasis with endobronchial tuberculosis. Forty per cent of the surgical specimens in the present study met criteria of the present writers for tuberculous bronchiectasis.

The experience with the present series of cases emphasizes that areas of tuberculous pneumonia and of cavitation are the areas in which bronchiectasis is most likely to be subsequently discovered.

It was found that the combination of bronchoscopy and bronchography was followed by a spread of the tuberculosis in 3 of 216 patients. Crellin and associates (5) encountered no post-bronchographic complications in a series of 503 cases. They referred to 16 delayed pulmonary complications in 220 consecutive bronchographies reported by Robertson and Forgan Morle (6).

The allergic reactions observed in the present study following bronchography were not of great magnitude. Twenty-one of the 26 consisted of only slight temperature elevation and subsided without therapy. Skin testing had not been done, but it is assumed that the 5 more serious reactions were due to iodism.

It was surprising that the small group of patients who left the hospital with considerable roentgenographic evidence of bronchiectasis had behaved subsequently so favorably from a clinical standpoint. This lends further support to the belief that the principal role of bronchography in pulmonary tuberculosis is to help determine the extent of tissue which should be resected surgically and not as a method to be used primarily as an indicator for surgery in asymptomatic patients.

SUMMARY

In pathologic specimens revealing bronchiectasis, the pre-surgical bronchogram had clearly delineated the bronchiectasis with 76 per cent accuracy. In pathologic specimens failing to reveal bronchiectasis, 20 per cent of the bronchograms had been over-interpreted as showing bronchiectasis. In only 40 per cent of the bronchiectatic specimens was it possible to make a diagnosis of tuberculous bronchiectasis.

Areas of pneumonic and cavitary tuberculosis have the highest incidence of associated bronchiectasis following antituberculous therapy. The incidence of post-bronchographic spread of tuberculosis was 1.2 per cent per bronchogram or 1.4 per cent per patient. There was a 10 per cent incidence of allergic reactions to iodized oil with only 1.5 per cent incidence of moderately severe reactions and no serious or fatal reactions. Oil retention hindered subsequent roentgenographic interpretation in 1.5 per cent of the patients.

With a short follow-up of an average of only nineteen months per patient, it was surprising to see that only 11 per cent of the patients had difficulty with respect to their tuberculosis when they had had extensive disease and extensive bronchiectasis at the time of discharge.

SUMARIO

El Estudio Broncográfico e Histopatológico Correlacionado de la Enfermedad Bronquial en 216 Tuberculosos

En ejemplares patológicos que revelaban bronquiectasia, el broncograma prequirúrgico ya había delineado netamente la bronquiectasia con una exactitud de 76 por ciento. En los que no revelaban bronquiectasia, en 20 por ciento se había exagerado la interpretación para

presuponer bronquiectasia. Solamente en 40 por ciento de los ejemplares bronquiectáticos fué posible hacer un diagnóstico de bronquiectasia tuberculosa.

Las zonas de tuberculosis neumónica y cavitaria muestran la mayor incidencia de bronquiectasia concomitante después de la terapéutica antituberculosa. La incidencia de difusión postbroncográfica fué de 1.2 por ciento por broncograma o 1.4 por ciento por enfermo. Hubo una incidencia de 10 por ciento de reacciones alérgicas al aceite yodado con una incidencia de no más de 1.5 por ciento de reacciones moderadamente intensas y sin ninguna reacción grave o letal. La retención del aceite impidió la subsiguiente interpretación roentgenográfica en 1.5 por ciento de los enfermos.

Con un breve período de observación subsecuente por término medio en cada enfermo, fué sorprendente observar que no más de 11 por ciento de los enfermos encontraron dificultad con respecto a su tuberculosis, habiendo tenido enfermedad difusa y bronquiectasia difusa al darlos de alta.

RESUME

Etude comparée bronchographique et histo-pathologique de la lésion bronchique chez 216 tuberculeux

Dans les pièces pathologiques révélant la bronchiectasie, le bronchogramme préopératoire avait clairement délimité la bronchiectasie avec une précision de 76%. Parmi les pièces pathologiques ne révèlant pas la présence de bronchiectasie, l'interprétation diagnostique de bronchiectasie était erronée dans 20% des cas. Le diagnostic de bronchiectasie tuberculeuse ne fut possible que dans 40% seulement des spécimens de bronchiectasies. A la suite du traitement antituberculeux, les segments affectés de lésions tuberculeuses pneumoniques et cavitaires ont l'incidence de bronchiectasie associée, la plus haute. L'incidence de la dissémination post-bronchographique était de 1,2% par bronchogramme soit 1,4% par malade. L'incidence des réactions allergiques à l'huile iodée était de 10%; 1,5% des réactions étaient de sévérité modérée, aucune n'a été sérieuse ou fatale. La rétention d'huile a ultérieurement prévenu l'interprétation radiologique chez 1,5% des patients.

Au cours d'une courte période de surveillance, en moyenne dix-neuf mois par malade, il a été constaté avec surprise que 11% seulement avaient eu des accidents en rapport avec leur tuberculose, alors qu'ils étaient porteurs de lésions extensives et de bronchiectasies extensives au moment où l'hospitalisation avait pris fin.

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THE SURGERY OF PULMONARY TUBERCULOSIS[1]

A Twelve-Year Experience

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INTRODUCTION

The past eight years have witnessed a transition in the surgery of pulmonary tuberculosis from an era of pneumothorax-thoracoplasty to one of chemotherapy-resection. Before presenting the experience of the writers with resection in pulmonary tuberculosis, surgical data which reflect the therapeutic transition just mentioned will be reviewed.

During the past twelve years (January 1, 1943, to January 1, 1955), the writers have performed 1,841 operations upon 1,363 patients (table 1). Because the sources of those patients have remained remarkably constant, a year by year comparison of the kinds of surgery performed reflects quite accurately the changes in the treatment of pulmonary tuberculosis in this part of the country over that period of time. Eighty per cent of the patients were referred by private physicians and were operated upon in a private hospital. Twenty per cent were cared for in two tax-supported hospitals. Females have outnumbered the males by a little more than three to two (table 2). The preponderance of female patients has decreased in the past few years, however, and last year the ratio was exactly one to one.

In 1943 and, indeed, until 1949, the majority of patients were treated with surgery of a minor nature, i.e., operations upon the phrenic nerve, the lysis or attempted lysis of pleuropulmonary adhesions, et cetera. But after 1949, as artificial pneumothorax was abandoned as a treatment for pulmonary tuberculosis, the use of minor surgery rapidly declined until at the present time it has reached almost a vanishing point (table 3).

The largest number of patients treated was in the year 1948, at a time when there had been no reduction in the number of patients having thoracoplasty or minor surgery, and to these was added a considerable number in whom pulmonary resection was performed because all other methods of treatment had failed. Since that year, there has been a steady decline in the number of patients who have surgery, and it is anticipated that that decline will continue.

At the beginning of this period of study, the modern extrapleural thoracoplasty had become a well-standardized procedure and had proved to be a very effective method of treating cavitary tuberculosis. The number of patients treated each year with this type of surgery remained remarkably constant until

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TABLE 1

SURGERY FOR PULMONARY TUBERCULOSIS

Surgery	Number of Patients			Number of Operations		
	Male	Female	Total	Male	Female	Total
Major	310	506	816	497	797	1,294
Minor	214	333	547	214	333	547
Total	524	839	1,363*	711	1,130	1,841

* Private hospital patients: 1,094, 80%; public hospital patients: 269, 20%

TABLE 2

SURGERY OF PULMONARY TUBERCULOSIS

Sex Ratio for All Patients

	1943	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954
Per cent females	59	60	57	67	67	63	60	70	65	60	54	50

TABLE 3

NUMBER OF PATIENTS WHO HAD SURGERY

	1943	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	Total
Total patients	98	101	110	114	145	148	136	118	96	108	99	90	1,363
Minor surgery	59	54	67	61	80	78	55	40	21	17	10	5	547
Thoracoplasty	38	41	35	37	35	38	38	30	9	5	7	2	315*
Resection	0	5	6	16	29	29	37	41	65	76	78	76	458†

* 772 operations

† 465 operations

the end of 1949. Since that time, it has been rapidly abandoned as an elective surgical procedure in the treatment of tuberculosis, even though, as in the case of artificial pneumothorax, it was given no trial with concomitant antimicrobial therapy.

PULMONARY RESECTION

Pulmonary resection was, in the days before tuberculosis chemotherapy, a very hazardous method of attacking pulmonary tuberculosis, with a morbidity of 40 to 50 per cent and a mortality of 25 to 30 per cent (1–4). Yet its use was growing because it offered the one remaining hope to a considerable number of patients whose disease had resisted all other methods of treatment. After streptomycin and the other antimicrobial drugs became available, the morbidity and mortality of pulmonary resection declined sharply. Use of the procedure rapidly increased as it was selected with increasing frequency in preference to artificial pneumothorax or thoracoplasty. A look at the yearly changes in the indications for its use emphasizes this point (table 4).

TABLE 4

INDICATIONS IN PER CENT OF PATIENTS WHO HAD RESECTIONS

Indications	Sex	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	Total
1. Diagnostic	M	0	0	0	0.0	0	8	22	16	7	8	5	8
	F	0	0	7	9.0	0	0	3	0	4	0	7	3
	T	0	0	6	7.0	0	2	7	5	5	4	6	5
2. Elective	M	0	0	0	12.5	50	25	22	52	53	70	73	55
	F	20	25	7	33.0	43	18	45	60	60	51	63	47
	T	20	16	6	28.0	45	21	40	58	58	60	67	50
3. Destroyed lung	M	0	0	0	12.5	0	8	11	5	4	0	0	4
	F	20	0	7	9.0	9	4	3	7	4	7	7	7
	T	20	0	6	10.0	7	5	5	6	4	4	4	5
4. Collapse failure	M	0	50	100	37.0	37	17	22	5	36	22	14	22
	F	0	0	50	5.0	9	22	10	22	18	30	14	18
	T	0	17	56	14.0	17	21	12	17	24	26	14	20
5. Bronchial disease and sequelae	M	0	50	0	38.0	13	42	23	22	0	0	8	11
	F	60	75	29	44.0	39	56	39	11	14	12	9	25
	T	60	67	26	41.0	31	51	36	14	9	6	9	20

Indications for resection: The indications for pulmonary resection have been arranged into five groups. First, *diagnostic;* second, *elective;* third, *destroyed lung;* fourth, *failure of collapse therapy;* and fifth, *bronchial disease and its sequelae.* Patients operated upon previous to an etiologic diagnosis (diagnostic) have remained fairly constant at approximately 6 to 8 per cent, especially since the Los Angeles county-wide survey in 1950.

The proportion of patients who had pulmonary resection as an elective procedure steadily and rapidly increased from 18 per cent in the years 1944 and 1945 to 67 per cent in 1954. Resection because of destroyed lung declined from around 9 per cent in the first four years of study to about 4 or 5 per cent since then. Between 9 and 29 per cent of resections were done because of the failure of previous collapse procedures, with an average for the eleven years of 20 per cent. The greatest change has been in the decrease in the resections done because of bronchial disease and its sequelae since the antimicrobial drugs became available. In 1944–1945, 64 per cent of the resections were for this indication, while in 1954 the value shrank to 9 per cent. For the entire period of study, one in every two resections was done electively, that is, some other procedure might have been used effectively; one in five was done for bronchial disease and its sequelae and collapse failure, respectively; and one in twenty for destroyed lung and patients with undiagnosed pathology, respectively.

Age and sex: The age of patients accepted for resection has varied between fourteen and sixty-seven, the largest number falling in the thirty- to thirty-nine-year group (table 5). The average age of the patients each year has remained about the same and has not reflected as yet the trend to the older age group which has been so characteristic of newly reported cases in the past few years (table 6).

TABLE 5

PULMONARY RESECTIONS FOR TUBERCULOSIS

Age and Sex

	Number of Operations	Age Years					
		10–19	20–29	30–39	40–49	50–59	60–69
Male	159	5	27	44	54	27	2
Female	306	3	81	108	79	34	1
Total	465	8	108	152	133	61	3

TABLE 6

PULMONARY RESECTIONS FOR TUBERCULOSIS

Average Age of Patients

	Sex	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	Average
Segmentals and lobectomies	M			30	39	30	32	47	44	38.0	42	36–	38–
	F	31	31	22	40	30	36	35	37	36.0	37	35.0	34–
	T	31	31	25	40	30	35	38	39	36.0	39	35.6	34.5
Pneumonectomies	M		39	42	37	41	39	41	32	45.0	39	47.0	40.0
	F	38	35	34	41	34	39	38	40	37.0	37	30.0	37–
	T	38	37	35	40	36	39	39	39	38.5	38	35.5	38–
Total resections	M		39	36	38	35	36	45	43	38.0	41	36.7–	39–
	F	37	34	32	41	33	38	36	38	36.0	36	34.7	36–
	T	37	36	33	40	34	37	38	40	36.0	39	35.6	37–

TABLE 7

PULMONARY RESECTIONS FOR TUBERCULOSIS

Sex vs. Extent of Resection

	Less Than One Lobe	One Lobe	One Lobe Plus	One Lung	Total
Number of operations	90	189	47	141	465.0
Number of males	45	65	15	34	159.0
Number of females	45	122	32	107	306.0
Per cent of females	50	65	68	76	65.8

The preponderance of female patients has been greater among patients having resections than among patients as a whole, i.e., 65.8 per cent as against 61.6 per cent. But in the past few years the experience has reflected to a limited degree an increasing proportion of newly reported tuberculosis in male patients, and in 1954 pulmonary resection was performed upon almost as many males as females. In the experience of the present writers, female patients had disease requiring the resection of more lung tissue than the male patients. Of the pneumonectomies, females outnumber the males by three to one, while among patients having

TABLE 8

PULMONARY RESECTIONS FOR TUBERCULOSIS

Variation in Extent of Resection by Year

Extent of Resection	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	Total
Segments	0	0	0	0	1	0	2	3	31	19	34	90
Lobes	1	1	3	4	9	15	20	40	30	34	30	187
Lobe plus	0	0	0	1	1	3	6	10	10	10	6	47
Lung	4	5	13	24	18	20	13	12	6	17	9	141
Total	5	6	16	29	29	38	41	65	77	80	79	465

TABLE 9

PULMONARY RESECTIONS FOR TUBERCULOSIS

Operative Mortality *vs.* Extent of Resection

Extent of Resection	Number of Operations	Mortality		
		Early (10 Days)	Late	Total
Less than one lobe	90	1 (1.1%)	0 (0)	1 (1.1%)
One lobe	187	1 (0.5%)	1 (0.5%)	2 (1.1%)
One lobe plus	47	1 (2.2%)	0 (0)	1 (2.2%)
One lung	141	10 (7.0%)	10 (7.0%)	20 (14.0%)
Total	465	13 (2.8%)	11 (2.4%)	24 (5.2%)

segmental resections the ratio is exactly one to one. But, in spite of the great extent of their disease, the female patients have withstood surgery somewhat better than the males, having a mortality rate of 4.6 per cent as compared to 6.2 per cent for the males (table 7).

Extent of resection: As might be expected, the extent of resection required has decreased over the period of study (table 8). The cases have been placed in four categories: (*1*) those requiring less than one lobe (which includes a few wedge resections); (*2*) the lobectomies; (*3*) the lobes plus (a single lobe plus one or more segments, but less than one lung); and (*4*) complete pneumonectomy.

Operative mortality: The operative mortality has varied with the extent of resection. Operative mortality in the present report includes not only the deaths which occurred within the first ten postoperative days (classified as early), but also all deaths which occurred later from some complication resulting from the operation (classified as late). The causes of the later mortality are for the most part incident to the disease itself rather than to the surgical technique (table 9). The early operative mortality in those patients requiring resection of less than one lobe was 1.1 per cent; in those having one lobe resected, 0.5 per cent; one lobe plus, 2.2 per cent; and one lung, 7 per cent. This gives an over-all early operative mortality in 465 resections of 2.8 per cent. The late operative mortality was zero in the less than one lobe group; 0.5 per cent in the one lobe group; zero

TABLE 10

INFLUENCE OF SIDE RESECTED

Lobectomies (Including Segments)

	Male			Female			Total		
	Number of Resections	Number Died	Per Cent Mortality	Number of Resections	Number Died	Per Cent Mortality	Number of Resections	Number Died	Per Cent Mortality
Right	70	2	2.9	107	1	0.9	177	3	1.7
Left	55	1	1.8	92	0	0.0	147	1	0.7
Total	125	3	2.4	199	1	0.5	324	4	1.2
Pneumonectomies									
Right	19	6	31.6	44	6	13.6	63	12	19.0
Left	15	1	6.7	63	7	11.1	78	8	10.3
Total	34	7	20.6	107	13	12.1	141	20	14.2
Total Resections									
Right	89	8	8.9	151	7	4.6	240	15	6.2
Left	70	2	2.9	155	7	4.5	225	9	4.0
Total	159	10	6.3	306	14	4.6	465	24	5.2

in the one lobe plus group; and 7 per cent in the one lung group. The total operative mortality was 1.1 per cent in the less than one lobe group; 1.1 per cent in the one lobe group; 2.2 per cent in the one lobe plus group; and 14 per cent in the one lung group. The over-all operative mortality for the 465 patients was 5.2 per cent.

The mortality rate was greater in patients having resection on the right side (6.2 per cent) than on the left side (4.0 per cent). This was true in the case of lobectomies as well as pneumonectomies, but the difference is much greater in male patients when compared with female patients (table 10).

The influence of the antimicrobial drugs on both morbidity and mortality is much greater than any other factor. The patients have been divided into five categories: (*1*) those having resections before streptomycin; (*2*) diagnostic resections in patients who for the most part received no streptomycin; (*3*) those who had tubercle bacilli which were streptomycin-resistant; (*4*) those who received short-term treatment with antimicrobial drugs (three months or less of total pre- and postoperative treatment); and (*5*) those having more than a total of three months' treatment with antimicrobials. These five categories have been further divided into groups showing the extent of resection. These data are presented in table 11. The data may be summarized by saying that, generally speaking, the influence of the antimicrobial drugs, while not without significance in reducing the early operative mortality, was particularly important in reducing the late operative mortality. The experience over the past four years in operating upon 236 patients has shown an early operative mortality of 1.2 per cent and a later operative mortality of 0.4 per cent, or a total of 1.7 per cent. There is one

TABLE 11

PULMONARY RESECTIONS FOR TUBERCULOSIS

Influence of Antimicrobial Drugs

Use of Antimicrobial Drugs	Extent of Resection*	Number of Operations	Complications		Operative Mortality					
					Early		Late		Total	
			Number	Per Cent	Number	Per Cent	Number	Per Cent	Number	Per Cent
Before streptomycin	1	1	1	100.0	0	0.0	0	0.0	0	0.0
	2	8	0	0.0	0	0.0	0	0.0	0	0.0
	3	2	1	50.0	0	0.0	0	0.0	0	0.0
	4	39	11	29.0	2	5.0	6	15.0	8	21.0
Total		50	13	26.0	2	4.0	6	12.0	8	16.0
Diagnostic, no streptomycin	1	6	0	0.0	0	0.0	0	0.0	0	0.0
	2	11	2	18.0	1	9.0	0	0.0	1	9.0
	3	2	0	0.0	0	0.0	0	0.0	0	0.0
	4	3	1	33.0	0	0.0	1	33.0	1	33.0
Total		22	3	13.0	1	4.5	1	4.5	2	9.0
Streptomycin resistant	1	0	0	0.0	0	0.0	0	0.0	0	0.0
	2	1	1	100.0	0	0.0	1	100.0	1	100.0
	3	0	0	0.0	0	0.0	0	0.0	0	0.0
	4	8	8	100.0	1	12.5	2	25.0	3	37.5
Total		9	9	100.0	1	11.0	3	33.0	4	44.0
Short-term streptomycin (less than 3 months)	1	3	0	0.0	0	0.0	0	0.0	0	0.0
	2	80	6	7.5	0	0.0	0	0.0	0	0.0
	3	18	1	5.5	1	5.5	0	0.0	1	5.5
	4	59	9	15.0	5	8.3	0	0.0	5	8.3
Total		160	16	10.0	6	3.7	0	0.0	6	3.7
Long-term streptomycin (more than 3 months)	1	80	8	10.0	1	1.2	0	0.0	1	1.2
	2	87	3	3.4	0	0.0	0	0.0	0	0.0
	3	25	1	4.0	0	0.0	0	0.0	0	0.0
	4	32	6	19.0	2	6.2	1	3.1	3	9.4
Total		236	18	7.6	3	1.2	1	0.4	4	1.7

* 1. Segments 2. Lobes 3. Lobes plus 4. Lungs

point which should be stressed, namely, that diagnostic resections are not without hazard. In the present series the group of 22 patients with diagnostic resections includes all those in whom a logical diagnosis was not established prior to thoracotomy. One early and one late death occurred which give an over-all operative mortality of 9 per cent. This compares quite unfavorably with 1.7 per cent in patients who had long-term antimicrobial drugs. It is the present

TABLE 12

USE OF SPACE-REDUCING OPERATIONS

Space-Reducing Operation	Lobes and Segments		Lungs		Total	
	Number	Per Cent	Number	Per Cent	Number	Per Cent
Previous thoracoplasty	14	4.3	32	22.7	46	9.9
Concomitant thoracoplasty	102	31.5	21	14.9	123	26.5
After resection thoracoplasty	15	4.6	41	29.1	56	12.0
Total thoracoplasties	131	40.4	94	66.7	225	48.4
Phrenicectomy or pneumoperitoneum	15	4.6	0	0.0	15	3.2
None	178	55.0	47	33.3	225	48.4
Total resections	324	100.0	141	100.0	465	100.0

policy of the writers to try to protect all diagnostic thoracotomies when tuberculosis is suspected as the diagnosis by giving antimicrobial drugs for a preoperative period of at least one week. Experience in the present series with patients with streptomycin-resistant infections has been especially bad, serious complications having developed in all 9 of them, with a total operative mortality of 44 per cent. It should be said, however, to the credit of our medical colleagues, that almost all of these patients were seen in the early days of streptomycin therapy.

Supplementary space-reducing procedures: Use of space-reducing operations has varied over the years (table 12). Approximately 10 per cent of all resections in the present series were in patients who had had a previous thoracoplasty. A concomitant thoracoplasty was done in approximately 26 per cent and a thoracoplasty in the early postoperative period in an additional 12 per cent. Thus, the number of patients who received no collapse procedure was approximately the same as the number of patients who had thoracoplasties (48 per cent). In approximately 3 per cent of the cases only was a phrenic operation or pneumoperitoneum performed. About 2 per cent of the patients having no space-reducing procedure have required a thoracoplasty later because of some complication.

In the early days of pulmonary resection the writers did not usually perform thoracoplasty at the time of the resection and performed one later only if there appeared a definite indication. In the middle period, because of increasing reports in the literature of reactivations or other serious complications in patients not having space-reducing operations, the use of thoracoplasty was increased. Follow-up studies among the patients who had no collapse procedures, however, as compared with those who had had the additional procedures, have continued to justify an opinion expressed earlier (2) that with the proper postoperative care there is little danger of spread or reactivation of disease because of overexpansion of remaining lung tissue. Moreover, no particular ill effects have been seen of pulmonary emphysema in patients who had no emphysema before surgery. The present policy is to do a modified thoracoplasty in all patients having a complete pneumonectomy and in those patients having less than one lung removed, if it appears at the time of surgery that the remaining lung will not fill the

pleural cavity. In those patients having any degree of pulmonary emphysema, the thoracic cage is tailored to fit the remaining lung tissue by means of a modified thoracoplasty.

BARLOW SANATORIUM PATIENTS

Of the 465 resections, 190 have been performed upon patients from the Barlow Sanatorium. This institution has an excellent follow-up of its patients and for that reason only the patients from that institution are included in the follow-up study. There were no early operative deaths among the 144 patients who had lobectomies, and only one in the 45 pneumonectomies, giving an early operative mortality of 0.5 per cent (table 13). There was one late operative death among the patients who had lobectomies, and two among the patients who had pneumonectomies, making a late operative mortality of 1.6 per cent, and an over-all operative mortality of 2.1 per cent.

For purposes of comparison, the follow-up studies of the patients who had resections are presented along with similar studies among those patients who had therapeutic thoracoplasties during the past twelve years. The operative mortality in 120 patients who had 284 thoracoplasty stages was 0.7 per cent, as compared to 2.1 per cent among the pulmonary resection patients. Thus, it is seen that the operative hazard in pulmonary resections has been a little higher than in thoracoplasty (table 14).

Sputum "conversion" in the two groups shows interesting differences (table 15). By sputum "conversion" is meant the failure to demonstrate tubercle bacilli by culture and animal inoculation of pooled material from a seventy-two-

TABLE 13

BARLOW SANATORIUM RESULTS

	Number of Operations	Operative Mortality		
		Early	Late	Total
Segments	44	0	0	0
Lobes	84	0	1 (1.2%)	1 (1.2%)
Lobe plus	17	0	0	0
Total lobes	145	0	1 (0.7%)	1 (0.7%)
One lung	45	1 (2.2%)	2 (4.4%)	3 (6.7%)
Total resections	190	1 (0.5%)	3 (1.6%)	4 (2.1%)

TABLE 14

BARLOW SANATORIUM RESULTS
Resections Compared with Thoracoplasties

Operation	Number of Patients	Number of Operations	Per Cent Female	Operative Mortality		
				Early	Late	Total
Resection	188	190	68	1 (0.5%)	3 (1.6%)	4 (2.1%)
Thoracoplasty	120	284	62	0	2 (0.7%)	2 (0.7%)

TABLE 15

BARLOW SANATORIUM FOLLOW-UP STUDY
Reversal of Infectiousness

	Thoracoplasties	Resections
Number with cultures negative for *M. tuberculosis*		
Within three months	57 (55.0%)	106 (71.0%)
Later without additional treatment	18 (17.4%)	16 (10.5%)
With additional drugs or surgery	10 (9.6%)	19 (12.5%)
Total	85 (82.0%)	141 (94.0%)
Number with cultures positive for *M. tuberculosis*		
Intermittently	11 (10.6%)	1 (0.7%)
Continuously	6 (5.8%)	3 (2.0%)
Late (relapse)	2 (1.6%)	5 (3.3%)
Total followed	104 (100.0%)	150 (100.0%)

hour sputum collection and from an early morning gastric lavage. Early sputum "conversion" was higher in the resection group (71 per cent) than in the thoracoplasty group (55 per cent), but a larger number of the thoracoplasty patients showed late "conversion" without additional treatment (17 per cent) than did the resection group (10.9 per cent). The addition of more surgery or drug therapy was required in 12.5 per cent of the resection group in order to obtain sputum "conversion," and in only 10 per cent of the thoracoplasty group. It should be pointed out, of course, that most of the patients having resection who needed additional drug therapy were those in the early period when the drugs were given for only a comparatively short time (less than three months).

Of the 150 patients who had pulmonary resection between the years 1943 and 1953, inclusive, and were followed for a period of one to eleven years, 131 (94 per cent) now have sputum negative for tubercle bacilli; and of the 104 thoracoplasty patients followed for the same length of time, 85 (82 per cent) now have a comparable noninfectious status. A fairly large number of thoracoplasty patients (10 per cent) intermittently discharge tubercle bacilli in the sputum, but are apparently well. Only one patient (0.7 per cent) in the resection group is in this category. Tubercle bacilli have persisted in the sputum of 5.8 per cent of the thoracoplasty patients since surgery, whereas only 2 per cent of the resection group have failed to "convert." Approximately the same proportion of patients in each group has shown bacteriologic relapse. Approximately 2 per cent of the thoracoplasties and 3 per cent of the resections are included in this group.

It should be remembered in comparing these results that none of the thoracoplasty patients had had treatment with the antimicrobial drugs before their surgery, and only a comparatively few (12 patients) had drug therapy later. Of these 12, 8 also had pulmonary resections following the failure of the thoraco-

TABLE 16

BARLOW SANATORIUM FOLLOW-UP STUDY
Present Condition

	Thoracoplasties	Resections
Living		
Well		
With no additional treatment	81 (73.7%)	127 (78.9%)
With additional drugs	9 (8.2%)	19 (11.8%)
With additional drugs plus surgery	5 (4.5%)	3 (1.8%)
Total well	95 (86.4%)	149 (92.5%)
Sick	10 (9.1%)	6 (3.7%)
Total living	105 (95.5%)	155 (96.2%)
Dead		
Operative deaths		
Early	0 (0)	1 (0.6%)
Late	2 (1.8%)	3 (1.9%)
Nonoperative		
From tuberculosis	2 (1.8%)	0 (0)
From other causes	1 (0.9%)	2 (1.3%)
Total followed	110 (100.0%)	161 (100.0%)

plasty. As might be expected, the results in the resecton of the thoracoplasty failures were not too good. Two patients (25 per cent) failed to survive surgery, one is still ill with tuberculosis, and 5 are living and well with sputum negative for tuberculosis.

The present state of health of patients having the two different surgical treatments shows fewer differences than might be expected (table 16). Of the 120 thoracoplasty patients, the present condition of 110 is known. Five are dead (4.5 per cent) and 105 are living. Of the 105 living, 95 (86.3 per cent of the total followed) are well; and 10 (9.1 per cent) are still sick. Of the 165 resection patients, the present condition of 161 is known. Six (4 per cent) are dead; and 155 (96 per cent) are living. Of the living, 149 (92.5 per cent), are well and 6 (3.5 per cent) are still ill with tuberculosis.

It is thus seen that, while thoracoplasty compares quite favorably with pulmonary resection so far as percentage of living patients is concerned, it compares less favorably in so far as the total percentage of patients who are living, well, and have sputum negative for tubercle bacilli is concerned.

DISCUSSION

In the last twelve years there has been a transition in the therapy of pulmonary tuberculosis from a pneumothorax-thoracoplasty era to a drug-resection era. This transition occurred, not because of the failure of pneumothorax-thoracoplasty treatment, but because of many other factors. Among these were the improved mortality and morbidity rates incident to the use of the antimicrobial

drugs in resectional surgery; the desire on the part of both the patients and doctors to remove foci of caseonecrotic disease which might endanger the future welfare of the patient; the decrease in the treatment time which resection appeared to offer; and the dislike on the part of patients for the long-continued "needling" of pneumothorax and the multi-stage and deforming features of thoracoplasty. There are indications that the comparatively high prevalence of surgical treatment is now diminishing and that the future will see less need for the surgical treatment of tuberculosis.

In the late nineteen forties and early nineteen fifties, there was a rather large reservoir of treatment-failure patients who were susceptible to further curative efforts with the antimicrobial drugs and pulmonary resection. This reservoir has now been largely eliminated, and the task of the surgeon in the coming era would appear to be to treat a diminishing proportion of drug failures, to eradicate the "burned out" or destroyed lung tissue which is left after patients have had their tuberculosis brought under control, and to try to help salvage a small number of patients with chronic bilateral disease who will continue to be seen. This outlook must be modified by the possibility that inadequate and therefore unsuccessful drug therapy may produce a considerable number of patients who, by virtue of a persistence of active tuberculous foci containing drug-resistant organisms, will offer a larger and more difficult problem to both chest physician and surgeon.

SUMMARY

Data concerning 1,841 operations upon 1,363 tuberculous patients are presented which reflect the changes in the therapy of pulmonary tuberculosis which have occurred during the past twelve years.

The increasing morbidity rates of pulmonary tuberculosis among the male population is just beginning to show in the present surgical statistics. The increasing incidence of tuberculosis in the older age groups has not yet been reflected in surgical experience.

The changes in the proportion of patients who have had pulmonary resections during the twelve-year period for indications specified in Barlow Sanatorium reflect a marked lessening of bronchial disease as a serious problem in pulmonary tuberculosis and a reduction of the reservoir of pulmonary disease due to the sequelae of tuberculous bronchitis. The data also reflect the preference for pulmonary resection over collapse therapy which has occurred since the advent of the antimicrobial drugs has made resection a comparatively safe procedure.

In the experience of the writers, female patients have had disease which required more extensive resection than males, but they have withstood surgery better. Operative mortality is greater as the extent of resected pulmonary tissue increases, but the difference between segmental resection and lobectomy is not nearly so great as between lobectomy and pneumonectomy. Operations upon the right lung carry a greater hazard than upon the left, both in lobectomy and pneumonectomy.

The antimicrobial drugs have been the most important factor in reducing operative mortality and morbidity in pulmonary resections, and the improve-

ment has been much greater in the late operative period (after ten days post-operatively) than in the early period.

Careful follow-up studies upon 167 patients from the Barlow Sanatorium who had resections between January 1, 1944, and January 1, 1953, showed reversal of infectiousness (sputum "conversion") in 94 per cent of 150 patients followed by yearly examinations and also show that 92.5 per cent of 161 patients followed for at least a year are living and well. Similar studies of 120 patients who had therapeutic thoracoplasties during the same period of time show 82 per cent with reversal of infectiousness and that 86.4 per cent are living and well. When survival rates are compared, the differences are not so great: 96.2 per cent of the patients who had resections are alive as compared with 95.4 per cent of those who had therapeutic thoracoplasties.

SUMARIO

La Cirugía de la Tuberculosis Pulmonar: Doce Años de Experiencia

Preséntanse aquí datos relativos a 1,841 operaciones en 1,363 tuberculosos, que reflejan las modificaciones que ha experimentado la terapéutica de la tuberculosis pulmonar durante los últimos doce años.

El aumento en los coeficientes de morbidad de la tuberculosis pulmonar entre la población masculina apenas comienza a aparecer en nuestras estadísticas quirúrgicas. La creciente incidencia de la enfermedad en los grupos de edad más avanzada no se han reflejado aun en la esfera quirúrgica.

Las alteraciones observadas durante el docenio estudiado en la proporción de enfermos que han experimentado resecciones pulmonares por las indicaciones estipuladas en el sanatorio del A. reflejan una decidida baja de la enfermedad bronquial como problema grave en la tuberculosis pulmonar y una limpiadura del reservorio de afección pulmonar creado por las secuelas de la bronquitis tuberculosa. Reflejan además la preferencia gozada por la resección pulmonar sobre la colapsoterapia desde que el advenimiento de las drogas antimicrobianas ha convertido la resección en procedimiento comparativamente seguro.

Según ha observado el A., las mujeres han tenido enfermedad que requirió resección más extensa que los hombres, pero toleraron mejor la cirugía. La mortalidad operatoria es mayor a medida que aumenta el tejido pulmonar resecado, pero la diferencia no es con mucho tan considerable como entre una lobectomía y una neumonectomía. Las operaciones en el pulmón derecho entrañan mayor riesgo que en el izquierdo, tanto en la lobectomía como la neumonectomía.

Las drogas antimicrobianas han sido el factor más importante en la reducción de la mortalidad y la morbidad operatorias en las resecciones pulmonares, y el mejoramiento ha sido mucho mayor en el período operatorio tardío (más de diez días después de la operación) que en el temprano.

Cuidadosos estudios de observación subsecuente en 167 enfermos del Sanatorio Barlow a quienes se hicieron resecciones entre el 1° de enero de 1955 y el 1° de enero de 1953, muestran "viraje" del esputo en 94 por ciento de 150 enfermos seguidos por medio de exámenes anuales y muestran además que 92.5 por ciento de 161 enfermos seguidos a lo menos un año se hallan vivos y sanos. Estudios similares de 120 enfermos a quienes se hicieron toracoplastias terapéuticas durante el mismo período de tiempo mostraron en 82 por ciento "viraje" del esputo y que 86.4 por ciento se hallan vivos y sanos. Cuando se comparan los coeficientes de sobrevivencia, las diferencias no son tan grandes: 96.2 por ciento de los sujetos a quienes se hicieron resecciones se hallan vivos, comparados con 95.4 por ciento de los que experimentaron toracoplastias terapéuticas.

RESUME

La chirurgie de la tuberculose pulmonaire: Douze années d'expérience

Présentation de données concernant 1.841 opérations effectuées chez 1.363 tuberculeux et traduisant les changements survenus dans le traitement de la tuberculose pulmonaire dans ces douze dernières années.

L'accroissement des taux de morbidité de la tuberculose pulmonaire dans la population masculine commence seulement à apparaître dans nos statistiques chirurgicales. L'incidence croissante de la tuberculose dans les groupes d'âge avancé n'a pas encore été décelée dans notre pratique chirurgicale.

Les variations dans la proportion des malades ayant subi des résections pulmonaires pendant cette période de douze ans, pour les indications spécifiées dans notre sanatorium, reflètent la réduction marquée des lésions bronchiques comme facteurs de problèmes graves en tuberculose pulmonaire et le nettoyage des foyers de lésions pulmonaires dus aux séquelles de la bronchite tuberculeuse. Elles traduisent également la préférence donnée à la chirurgie d'exérèse sur la collapsothérapie, qui s'est manifestée depuis que l'introduction des agents antibactériens a rendu l'exérèse un procédé relativement sûr.

Selon l'expérience de l'auteur, les femmes présentaient des lésions nécessitant des résections plus étendues que chez l'homme, mais elles supportaient mieux les interventions. La mortalité est plus élevée à mesure que l'étendue du tissu pulmonaire réséqué augmente, mais la différence est loin d'être aussi marquée qu'entre une lobectomie et une pneumonectomie. Les interventions sur le poumon droit dans la lobectomie ou la pneumonectomie entraînent plus de risques que celles pratiquées sur le poumon gauche.

Les agents antibactériens constituaient le facteur le plus important dans la réduction de la mortalité et de la morbidité des résections pulmonaires; et le progrès a été encore beaucoup plus sensible dans la période opératoire tardive (dix jours après l'intervention chirurgicale) que dans la période initiale.

Une étude minutieuse de l'observation de 167 malades du Sanatorium Barlow, qui avaient été soumis aux résections entre le 1er janvier 1944 et le 1er janvier 1953, a montré la "conversion" du crachat chez 94% des 150 malades examinés annuellement et également que, sur un nombre de 161 patients suivis pendant au moins un an, 92,5% d'entre eux étaient vivants et se portaient bien. Des recherches similaires effectuées chez 120 patients, ayant été soumis à la thoracoplastie thérapeutique pendant cette même période de temps, montrent chez 82% d'entre eux la "conversion" de l'expectoration; 86,4% étaient vivant et se portaient bien. La comparaison des taux de survie montre que les différences ne sont pas si grandes: 96,2% des malades soumis au résections sont vivants par comparaison avec 95,4% pour ceux soumis a la thoracoplastie thérapeutique.

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PYRAZINAMIDE-ISONIAZID[1,2]

Comparison with Isoniazid–Para-Aminosalicylic Acid in Active Pulmonary Tuberculosis with the Choice of Regimens Determined by Chance

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INTRODUCTION

In a previous study at this hospital (1), the drug pyrazinamide was found to be of short-term benefit in the treatment of pulmonary tuberculosis in agreement with the original observations of Yeager and his associates (2–5). Moreover, the use of pyrazinamide-isoniazid at this hospital (1) also yielded therapeutic results which were in agreement with those of other investigators (6–8). In all of the above clinical investigations, however, one or more instances of jaundice occurred during the chemotherapy. Indeed, the group of investigators whose studies in experimental animals and man had led them to the conclusion that pyrazinamide-isoniazid was the most powerful of the available tuberculosis chemotherapies (7, 9, 10) warned against its routine use in pulmonary tuberculosis because of hepatic toxicity. This serious view of the toxicity of pyrazinamide was not completely accepted by some of the other investigators (8, 10). Thus, although there was little doubt of the effectiveness of pyrazinamide-isoniazid in the treatment of tuberculosis, there was doubt about the gravity of its toxicity. This posed the question whether the toxicity of pyrazinamide-isoniazid was sufficiently serious to negate the promise of superior results in human tuberculosis. Or, to put it differently, were the results which could be obtained with pyrazinamide-isoniazid so markedly superior that it would be justifiable to regard a certain relatively low incidence of hepatotoxicity as tolerable? These questions seemed especially important because the pyrazinamide-isoniazid regimen was fully active in patients with streptomycin-resistant tubercle bacilli. Indeed, the use of pyrazinamide with streptomycin in streptomycin-susceptible infections had been favorably reported (11).

In an attempt to answer this question, a randomized study was begun at the Veterans Administration Medical Teaching Group (Kennedy) Hospital, in which pyrazinamide-isoniazid were compared in efficacy and toxity with isoniazid–para-aminosalicylic acid (PAS). This latter regimen was chosen because it had been found to be about the equal of any regimen routinely used in the treatment of tuberculosis (12) and because, theoretically, the only variables such a comparison contained would be the pyrazinamide and the PAS.

[1] From the Pulmonary Disease Division, Medical Service, Veterans Administration Medical Teaching Group (Kennedy) Hospital, Memphis 15, Tennessee.

[2] A preliminary report on the progress of this study was read at the Fourteenth Veterans Administration, Army, and Navy Conference on the Chemotherapy of Tuberculosis, Atlanta, Georgia, February 7–10, 1955.

PLAN OF INVESTIGATION

The criteria for admission to the study were the presence of active pulmonary tuberculosis, sputum positive for *M. tuberculosis*, and the absence of prior treatment with isoniazid or pyrazinamide. Previous treatment with PAS was permitted if the bacilli had not become PAS-resistant. The patients with odd hospital numbers received pyrazinamide-isoniazid; those with even numbers received isoniazid-PAS. Because of the hepatotoxicity of pyrazinamide, however, patients with preliminary bromsulphalein retentions greater than 7 per cent were removed from the study. In all other respects, the patients were treated identically with respect to bed rest, diet, and indications for collapse therapy or surgery, which were the same as were afforded all patients with pulmonary tuberculosis at the hospital.

Composition and comparability of treatment groups: A total of 93 patients, all male, has thus far been treated for a minimum period of four months. Thirty-eight patients received pyrazinamide-isoniazid and 55 received isoniazid-PAS. The discrepancy in numbers is due to "the luck of the draw" in a large general hospital as well as the exclusions due to deviations from the normal in the preliminary liver function tests in some patients who were otherwise eligible. To check the comparability of the resulting two groups of patients, they were analyzed according to age, stage of disease, and race. A distinction was made also between those patients who received specific drug treatment for the first time and the re-treatment cases.

A slightly greater proportion of the patients who received pyrazinamide-isoniazid were in the fifth, sixth, and seventh decades (53 per cent) than were those who received isoniazid-PAS (47 per cent) (figure 1). More patients with far advanced disease received PAS (38 per cent) than pyrazinamide (31 per cent). The proportion of patients with minimal disease in each group was essentially the same (figure 2). A greater proportion of Negro patients received PAS (39 per cent) than pyrazinamide (32 per cent) (figure 3). Twenty of the 38 patients who received pyrazinamide (53 per cent) were re-treatment cases, whereas 26 of the 55 patients who received PAS (47 per cent) were re-treatment cases (figure 4). Thus, although there are minor differences between the two groups, these are not sufficiently important to indicate that one group was in any less favorable condition than the other at the start of therapy.

Collapse or resectional therapy: Of the 93 patients in the study, thus far only 12 have required any form of collapse therapy or resectional surgery. Four of these patients (11 per cent) were receiving pyrazinamide, whereas the remaining 8 patients (15 per cent) were receiving PAS. The types of additional therapy used on these patients are listed in table 1. The usual indications for additional measures at this hospital are either open cavity or

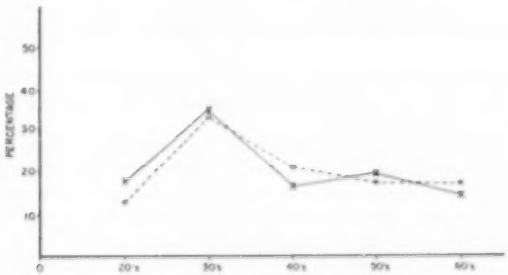

FIG. 1. Distribution of patients treated with pyrazinamide-isoniazid and isoniazid-PAS by age groups.

Pyrazinamide-isoniazid patients ○ - - - ○
Isoniazid-PAS patients × —— ×

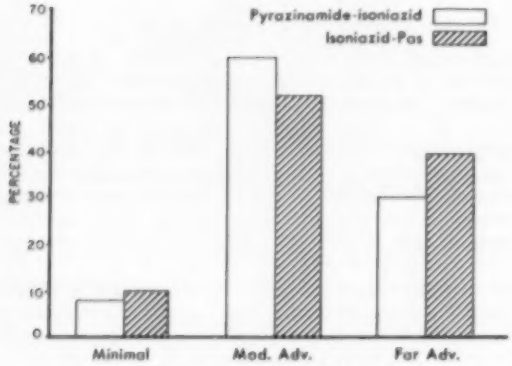

FIG. 2. Stage of disease by groups

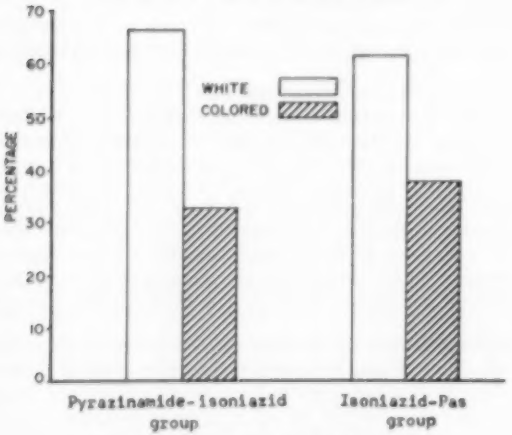

FIG. 3. Comparison by racial groups

sputum positive for *M. tuberculosis*, or both, at the end of six to eight months of chemotherapy.

Drug regimen: The doses of drugs used were uniformly as follows: isoniazid, 100 mg. three times daily; pyrazinamide, 750 mg. four times daily; and PAS (usually in the form of the sodium salt), 4 gm. three times daily.

Frequency of observations: Each patient's general condition, chest roentgenograms, sputum examination, and laboratory data were reviewed each month but, for the purpose of clarity without distortion, the pertinent findings at the end of four, eight, and twelve months only will be reviewed. Mention will also be made of a few patients who were treated under hospital supervision for eighteen months, although these cases were too few for valid comparison. The main reason why so few patients reached the full eighteen months of treatment originally projected was the remarkable efficacy of the regimens employed. Many patients with reversal of infectiousness and closed cavities demanded their release from the hospital after a much shorter interval, to continue their treatment and convalescence at home under the supervision of their local physicians. When such patients had received pyrazinamide during their hospital stay, it was recommended that they receive PAS instead for the remainder of their treatment at home.

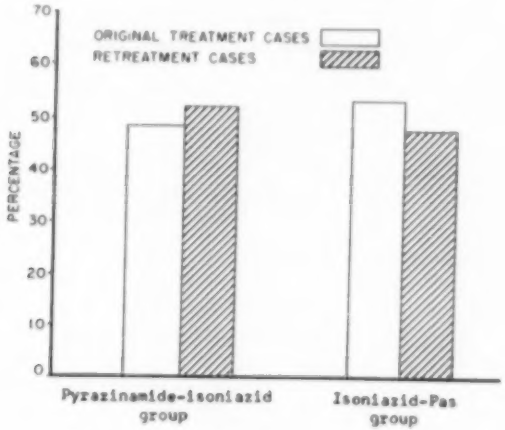

FIG. 4. Distribution of re-treatment cases.

TABLE 1

ADDITIONAL THERAPY EMPLOYED IN TWELVE PATIENTS

Procedure	Pyrazinamide and Isoniazid	Isoniazid and PAS
Pneumoperitoneum	1	3
Pulmonary resection	2	5
Plombage	1	2
Thoracoplasty	0	2
Number of patients who required procedures	4	8*

* Four of these patients required two procedures each.

Tests of hepatic function: All patients who received pyrazinamide had tests of liver function performed at monthly intervals, consisting of the cephalin flocculation and bromsulphalein tests and other laboratory procedures, such as the serum bilirubin and alkaline phosphatase, as indicated. In addition, a clinical alertness was maintained for the appearance of hepatitis.

OBSERVATIONS

At the end of four months' therapy (table 2), the proportion of patients showing substantial (moderate or marked) roentgenographic improvement was almost identical in both groups, 55 and 58 per cent. Similar proportions of patients in each group began to show sputum negative for *M. tuberculosis* on microscopy (82 and 87 per cent), but more of the patients on pyrazinamide-isoniazid had sputum which was "negative" on culture (82 per cent as compared with 67 per cent).

Two of the 55 patients receiving PAS showed some gastrointestinal intolerance for this drug during the first four months, and it was necessary to withhold the drug from these patients for two or three days on one or more occasions. Two of

TABLE 2

RESULTS OF THERAPY AT FOUR MONTHS

Drugs Administered	Number of Patients	Roentgenographic Improvement (Moderate or Marked)	Reversal of Infectiousness		Treatment Stopped (Toxicity)
			Concentrate	Culture	
Pyrazinamide-isoniazid	38	55% (21)	82% (31)	82% (31)	5% (2)
Isoniazid-PAS	55	58% (32)	87% (48)	67% (37)	0

Figures in parentheses represent actual number of patients

TABLE 3

RESULTS OF THERAPY AT EIGHT MONTHS

Drugs Administered	Number of Patients	Roentgenographic Improvement (Moderate or Marked)	Reversal of Infectiousness		Treatment Stopped (Toxicity)
			Concentrate	Culture	
Pyrazinamide-isoniazid	21	53% (12)	91% (19)	91% (19)	14% (3)
Isoniazid-PAS	37	57% (21)	89% (33)	87% (32)	0

Figures in parentheses represent actual number of patients.

the 38 patients receiving pyrazinamide developed complications that led to the discontinuation of treatment. One patient showed an idiosyncrasy to the periodic bromsulphalein injections by developing prompt urticarial reactions accompanied by generalized pruritis. The other patient complained of development of persistent numbness in the area of ulnar nerve distribution in the right hand. Although this was believed to be due to the isoniazid rather than to the pyrazinamide, this combination of drugs was discontinued when the numbness failed to respond to vitamins and other therapy. The toxic reactions in these 2 cases were not blamed on the pyrazinamide, although they are considered to be important as complications that may arise from the use of the pyrazinamide-isoniazid regimen.

The findings at eight months were somewhat similar (table 3) to those at the end of four months. The incidence of significant roentgenographic improvement was approximately the same in both groups (57 per cent compared with 53 per cent), and the rates of reversal of infectiousness of sputum on microscopy were almost identical (90 and 89 per cent). The rates of reversal of infectiousness of sputum on culture also were very similar (90 per cent compared with 86 per cent).

Three patients receiving pyrazinamide-isoniazid had treatment stopped because of toxicity, undoubtedly due to the pyrazinamide. One patient (E. L. M.) showed a sudden rise of bromsulphalein retention: to 51.5 per cent seven months after the start of therapy and to 73 per cent at nine months. At this time the pyrazinamide was discontinued, but a few days later the patient became jaundiced. He made a slow recovery.

TABLE 4

RESULTS OF THERAPY AT TWELVE MONTHS

Drugs Administered	Number of Patients	Roentgenographic Improvement (Moderate or Marked)	Reversal of Infectiousness		Treatment Stopped (Toxicity)
			Concentrate	Culture	
Pyrazinamide-isoniazid	8	6	7	7	2
Isoniazid-PAS	16	9	14	15	1

The second patient (J. C. C.) developed a sudden rise of bromsulphalein retention to 40.5 per cent six months after the start of therapy. The pyrazinamide was promptly discontinued, but this patient also became jaundiced within a few days. He improved rapidly and within two months his liver function tests had returned to normal. The third patient (O. E. C.) showed gradually rising bromsulphalein retention after the sixth month of therapy. When the retention reached 30 per cent during the eighth month, the pyrazinamide was discontinued. The patient remained clinically well and the bromsulphalein test gradually fell to 14 per cent just prior to discharge five months later.

Twenty-four patients were treated for one year or longer, 8 with pyrazinamide-isoniazid and 16 with isoniazid-PAS (table 4). The roentgenographic improvement appeared to be greater in those treated with pyrazinamide (75 per cent compared with 56 per cent), but the smaller number of patients available for comparison at one year makes this difference difficult to evaluate. The rate of reversal of infectiousness of sputum was identical, 87 per cent in each group. Two patients treated with pyrazinamide had to have treatment stopped because of toxicity. In one patient (C. H. C.), the bromsulphalein retention rose to 16 per cent during the twelfth month of therapy. The drug was discontinued and the patient was discharged on isoniazid-PAS treatment two weeks later. In the other patient (J. S. E.), bromsulphalein retention rose to 18 per cent after thirteen months of therapy. The pyrazinamide was discontinued, but treatment with isoniazid alone was maintained until the patient was discharged. One patient on PAS therapy developed nausea which required relief from therapy for a few days, and another patient developed persistent diarrhea which required cessation of further PAS therapy.

Only 6 patients were observed at eighteen months of therapy, 2 on pyrazinamide and 4 on PAS, and they are mentioned merely for the sake of completeness. The results were excellent in every way in the 2 patients who were treated with pyrazinamide-isoniazid, with significant roentgenographic improvement and reversal of infectiousness of sputum in both patients. On the isoniazid-PAS regimen, only 2 patients showed significant roentgenographic improvement and one of the 4 patients failed to attain reversal of infectiousness by culture. No valid interpretation of these results at eighteen months, however, can be permitted.

It should be mentioned that in the pyrazinamide-isoniazid group there was not a single incidence of emergence of an isoniazid-resistant strain of tubercle bacilli.

This was purported to be one of the chief advantages of the pyrazinamide-isoniazid combination (10), and is apparently supported by the current study. There was one case of the emergence of isoniazid resistance at five months on the isoniazid-PAS combination; however, the sputum immediately after this observation became consistently negative for *M. tuberculosis* so that this isolated finding could never be verified.

Comment: If it is permitted to draw any conclusions from this study, it would seem that the drugs used in both groups of patients were remarkably efficacious. The slight differences in therapeutic results have been noted and seem not too significant. From the more rapid reversal of infectiousness shown by the patients treated with pyrazinamide-isoniazid and the fact that a somewhat greater proportion of patients treated with isoniazid-PAS required collapse or resectional measures (15 per cent as compared with 11 per cent), then it might be concluded that pyrazinamide-isoniazid is slightly more effective.

Drug Toxicity

The question then becomes, Is the slight superiority in therapeutic effectiveness apparently shown by pyrazinamide-isoniazid in this study worth the payment exacted in the increased incidence and severity of toxicity? None of the patients in the isoniazid-PAS group developed any serious toxicity. Four of the 55 patients on this therapy developed gastrointestinal symptoms. In 3, these symptoms were temporary and responded to the withholding of PAS for two or three days or to a change in the form of the PAS prescribed. Only one patient had to be taken off further medication. This patient, after enduring PAS for twelve months, developed persistent diarrhea which did not completely improve until the PAS was permanently stopped. This was not considered to be a serious complication.

In marked contrast was the experience with pyrazinamide-isoniazid. Among the 38 patients, 2 developed jaundice and 3 others showed an increasing retention of the dye in the bromsulphalein tests, which seemed to indicate a developing drug hepatitis. In both patients who developed jaundice, there was a premonitory elevation of the bromsulphalein retention to more than 25 per cent forty-five minutes after the injection of 5 mg. of dye per kg. of body weight. In the first case, it did not seem that this progressively high retention was serious (8) and the treatment was continued because the patient failed to reveal any abnormal physical findings or to make any complaints. When, after nine months of treatment, an astounding bromsulphalein retention of 73 per cent was reported, the pyrazinamide-isoniazid was stopped. By that time, the serum bilirubin was 4.7 mg. per 100 ml. and the alkaline phosphatase and serum cholesterol had become elevated. In a few days the patient became obviously jaundiced. Slightly more than a year has passed and the patient appears and feels well, but the bromsulphalein retention is still 22 per cent.

The second patient, after approximately six months of therapy, suddenly showed a bromsulphalein retention of 40.5 per cent. Medication was promptly stopped despite the lack of symptoms. Apparently it was not stopped soon

enough to prevent the jaundice which occurred a few days later. This patient, however, recovered completely within six weeks, and the bromsulphalein test returned to normal.

The value of the bromsulphalein test in pyrazinamide hepatotoxicity having been established, therapy was discontinued in 3 other patients when it was found that the retention was repeatedly higher than 15 per cent. There was 30 per cent bromsulphalein retention in the first of these patients after nine months of therapy, and 16 and 18 per cent, respectively, after one year in the other 2 patients. These returned to normal some weeks after cessation of treatment.

The cephalin-cholesterol flocculation test, as devised by Hanger, indicates, when positive, hepatic parenchymal damage at the time of the test. According to Hoffman (13), it is considered to be the most reliable of all tests for liver injury in the presence of jaundice. It might be added that the experience of the writers with the cephalin flocculation test has been disappointing in indicating the onset, or even the presence, of pyrazinamide hepatitis, as the test did not become significantly abnormal in any of the patients who developed jaundice. This confirms the observation of Campagna and associates (8), whose three patients with jaundice had no significant elevation of the cephalin flocculation test. A similar experience also has been reported by Potter and Chang (11) who found that the cephalin flocculation test became positive in only 2 of their 8 patients who developed jaundice while being treated with pyrazinamide-isoniazid. The latter writers did not utilize the bromsulphalein dye retention test, which is believed to be the most reliable in foretelling the onset of jaundice induced by pyrazinamide. The latent hepatic toxicity of isoniazid which was described by Biehl and Nimitz (14), as a result of their production of marked fatty degeneration in the livers of dogs treated with high doses of isoniazid, may or may not be a potentiating factor in the incidence and severity of the pyrazinamide hepatitis complicating pyrazinamide-isoniazid therapy.

In the 2 cases of jaundice in the present report, the writers tried to obtain liver biopsies, but, because of the presence of emphysema in one patient and old pleural thickening and adhesions in the other, the risk of such a biopsy was considered unjustified by the consultant in the case. There seems to be little question, however, that the hepatitis was directly related to the medication the patients received.

DISCUSSION

The 2 cases of jaundice and the 3 other cases of increased bromsulphalein retention in the 38 patients who were treated with pyrazinamide-isoniazid appear definitely to confirm the high incidence of hepatitis reported by McDermott and co-workers (7). There were no fatalities in the present series. Previous mention has been made, however, of the death from fulminating hepatitis reported by McDermott and associates (7), and since then a few deaths from this cause have apparently occurred (11, 15). These fatalities refute the impression of Campagna and associates (8) that pyrazinamide-induced hepatitis is not serious.

Robinson and co-workers (16) described the liver pathology produced with

pyrazinamide experimentally in dogs. They found a zonal necrosis of the periportal areas, principally, and a dilatation of the lymphatics in the portal regions. The alkaline phosphatase and the icterus index were found to be elevated, indicating impairment of the hepatic excretory function (17). This perhaps may explain the value clinically of the bromsulfaphein dye retention test, which depends on the hepatic excretory function, in following patients under treatment with pyrazinamide prior to the appearance of jaundice. Once jaundice appeared, the serum bilirubin and the alkaline phosphatase seemed to be most useful tests to employ.

In the pyrazinamide-isoniazid series reported by Potter and Chang (7), there were 8 cases of hemoptysis, of which 7 were fatal, and Potter and Chang suggest that some relationship exists between the increased incidence of severe hemoptysis and the therapy employed. No fatal hemoptysis occurred in the patients of the present report who were treated with pyrazinamide-isoniazid, and the incidence of hemoptysis in the pyrazinamide-isoniazid group seemed to be about the same as in the isoniazid-PAS group. Thus, from their own experience, the writers cannot support the observation of an increased incidence and severity of hemoptysis in patients treated with pyrazinamide-isoniazid. Infrequently, it has been found that the prothrombin time has been prolonged, but this has been an inconsistent finding and has not been observed to be associated with hemoptysis or other frank bleeding.

The findings of this study suggest that pyrazinamide-isoniazid is a very effective combination of drugs in the treatment of active pulmonary tuberculosis, but that the combination offers little more than can be obtained, from a therapeutic standpoint, with a safer regimen such as isoniazid-PAS. Thus, the indications for the use of pyrazinamide-isoniazid in the treatment of tuberculosis appear to be limited to certain individual problems and to experimental studies. Valid reasons for the employment of pyrazinamide, despite its hepatotoxicity, might be the unavailability of any other potent antibacterial agent, such as streptomycin or PAS, for use in combination with isoniazid in the treatment of an advanced case of tuberculosis, or perhaps the need for an attempt to avoid surgery in a poor-risk patient. Even in such instances, treatment with pyrazinamide-isoniazid should not be undertaken unless adequate clinical and laboratory facilities are available for constant observations of patients and there is constant awareness of the frequency and occasional seriousness of toxic hepatitis.

SUMMARY

In a study of 93 patients with active pulmonary tuberculosis, 38 patients were treated with pyrazinamide-isoniazid for a minimum period of four months, compared with 55 patients treated similarly with isoniazid-PAS. The choice of treatment regimen was determined by random selection.

The pyrazinamide-isoniazid regimen may have been slightly superior because of a somewhat lesser need for additional measures, such as collapse or resectional therapy, and a somewhat more prompt disappearance of tubercle bacilli from the sputum.

There were 2 instances of complicating jaundice and 3 other cases of hepatitis among the 38 patients on pyrazinamide-isoniazid. No serious toxicity complicated the treatment with isoniazid-PAS, although one patient could not tolerate PAS for longer than one year.

Not a single patient treated with pyrazinamide-isoniazid excreted tubercle bacilli in the sputum which were resistant to isoniazid. One patient treated with isoniazid-PAS showed isoniazid-resistant organisms in one sputum specimen just prior to reversal of infectiousness.

The bromsulphalein dye retention test was found to be the most useful in detecting early evidence of hepatotoxicity. The cephalin flocculation test did not become significantly elevated in any of the patients in the present study.

It is believed that the incidence and severity of the hepatitis, which may complicate treatment with pyrazinamide-isoniazid, is probably too high a price to pay for the slight therapeutic superiority which may be obtained. Accordingly, the writers concur in the belief, expressed initially by others, that this regimen should not be used in routine forms of tuberculosis when other suitable agents are available.

SUMARIO

La Piracinamida-Isoniacida, Estudio en Enfermos Tomados al Azar y Comparación con la Isoniacida-PAS en la Tuberculosis Pulmonar Activa

En un estudio de 93 enfermos de tuberculosis pulmonar activa, tomados al azar, compáranse 38 pacientes tratados con piracinamida-isoniacida durante un período mínimo de cuatro meses con 55 tratados en forma semejante con isoniacida-PAS.

El régimen de piracinamida-isoniacida quizás fuera ligeramente superior dada la necesidad algo menor de usar otras medidas, tales como la colapsoterapia o la resección y el viraje algo más rápido del esputo en los cultivos.

Entre los 38 enfermos del régimen de piracinamida-isoniacida, hubo 2 casos de complicación por ictericia y tres casos más de hepatitis. Ninguna toxicidad grave complicó el tratamiento con isoniacida-PAS, aunque un enfermo no pudo tolerar el PAS más de un año. Ni un solo enfermo tratado con piracinamida-isoniacida manifestó en el esputo microbios resistentes a la isoniacida. Un enfermo tratado con isoniacida-PAS mostró en un ejemplar de esputo microbios isoniacidorresistentes precisamente antes de virar la infecciosidad.

La prueba de retención del colorante bromsulfaleína resultó ser la más útil para descubrir signos tempranos de hepatotoxicidad. La prueba de floculación de la cefalina no reveló mayor elevación en ninguno de los casos del estudio actual.

Opinan los AA. que la incidencia y la gravedad de la hepatitis, que puede complicar el tratamiento con piracinamida-isoniacida, constituyen probablemente un precio demasiado elevado para la leve superioridad terapéutica que quizás se obtenga, y convienen en la opinión, expresada antes por otros, que no debe usarse este régimen en las formas ordinarias de la tuberculosis cuando hay otros agentes apropiados disponibles.

RESUME

Pyrazinamide-Isoniazide, Étude d'un groupe non-sélectionné—Comparaison avec l'Isoniazide-PAS dans la tuberculose en activité

Dans une étude de 93 cas de tuberculose pulmonaire active choisis au hasard, 38 malades ayant été traités par l'association pyrazinamide-isoniazide pendant une période de quatre mois au minimum sont comparés à 55 malades traités de façon similaire avec l'isoniazide-PAS.

La thérapie pyrazinamide-isoniazide semble avoir été légèrement supérieure car elle a réclamé moins de thérapeutique adjuvante, telles que le collapsus ou la chirurgie d'exérèse, et la négativité des crachats en culture a été obtenue plus rapidement.

Il y a eu 2 cas d'ictère secondaire et 3 hépatites dans le groupe de 38 malades traité par la pyrazinamide-isoniazide. Aucune manifestation sérieuse d'intolérance n'a compliqué le traitement par l'isoniazide-PAS, toutefois, dans un des cas, le PAS n'a pas été toléré pendant plus d'un an. Aucun des malades traités par la pyrazinamide-isoniazide n'a présenté de germes résistants à l'isoniazide dans l'expectoration. Chez un malade traité par l'isoniazide-PAS, des germes isoniazide-résistants sont apparus dans un des spécimens de crachat immédiatement avant la disparition de la contagiosité.

L'épreuve de rétention de la bromosulfophtaléine a été trouvée des plus utiles pour déceler précocement l'atteinte toxique du foie. Dans aucun des cas étudiés dans cet article l'épreuve de floculation de la céphaline n'a eu de valeur significative.

Les auteurs estiment que la fréquence et la sévérité de l'hépatite qui peut compliquer le tratiement pyrazinamide-isoniazide sont des conséquences trop onéreuses par rapport à sa légère supériorité thérapeutique et ils sont d'accord pour penser, comme d'autres auteurs l'avaient déja exprimé, que ce mode de traitement ne doit pas être appliqué dans les formes habituelle de la tuberculose lorsque l'on dispose d'autres agents thérapeutiques.

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GROWTH OF MYCOBACTERIUM TUBERCULOSIS IN LIQUID MEDIA[1]

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INTRODUCTION

The heterogeneity of cell populations in mycobacteria and its relationship to virulence, colony morphology, staining characteristics, et cetera has been described by the classic work of Dubos and Davis (1). The use of Tween® 80 for the growth of virulent tubercle bacilli by Dubos and co-workers has permitted an abundant submerged dispersed growth in a simple medium. These workers pointed out that the ability to initiate growth from very small inocula is of little consequence when the aim is to produce large yields of bacilli growing in a dispersed manner. They also stressed that dispersed growth is necessary when both physiologic and chemical homogeneity of a culture are important. Since most metabolic and immunochemical studies require the use of large masses of cells, heterogeneity of populations complicates the analyses. The growth obtained on a liquid surface obviously is undesirable because of this heterogeneity

Dubos and Middlebrook (2) stated: "As in the case of other aerobic organisms, the final yield of tubercle bacilli in submerged cultures is limited by the rate of diffusion of oxygen through the liquid medium. It is obvious that increased oxygenation can be obtained by forced aeration or by mechanical agitation of the culture." Other investigators have shown the importance of molecular oxygen on growth and survival of the tubercle bacilli (3–7). However, it has been the experience of Kull and Grimm (8) and Schaefer, Marshak, and Burkhart (9) that agitation or aeration of *M. tuberculosis* in liquid culture hinders growth of this organism.

The purpose of this report is to present further evidence on the relation of agitation to the growth of *M. tuberculosis* in liquid media.

MATERIALS AND METHODS

The cultures, *Mycobacterium tuberculosis* var. *hominis*, strains 198ARB and H37Rv, were kindly supplied by Dr. Byron Olson of the National Institutes of Health and Dr. Sanford Elberg of the University of California, respectively. The virulence of 198ARB has been demonstrated for mice and guinea pigs (10). The organisms were maintained as stock cultures on a modified Löwenstein egg medium. The data presented were obtained with cultures which were transferred several times in liquid media until well-dispersed growth was obtained.

The liquid medium of Dubos and Middlebrook (2) used in these experiments had the following composition: 1.0 gm. KH_2PO_4, 6.8 gm. $Na_2HPO_4 \cdot 12H_2O$, 2.0 gm. asparagin, 2.0 gm. N. Z. Case[2], 1.0 ml. salts solution, 2.5 ml. glycerol, 0.5 ml. Tween® 80, and 1,000 ml. distilled water. The salts solution provided per liter of medium the following: 0.05 gm. ferric am.

[1] From the Medical Bacteriology Division, Headquarters Camp Detrick, Frederick, Maryland.

[2] N. Z. Case is an enzymatic digest of casein manufactured by Sheffield Farms Co., Inc. New York, New York.

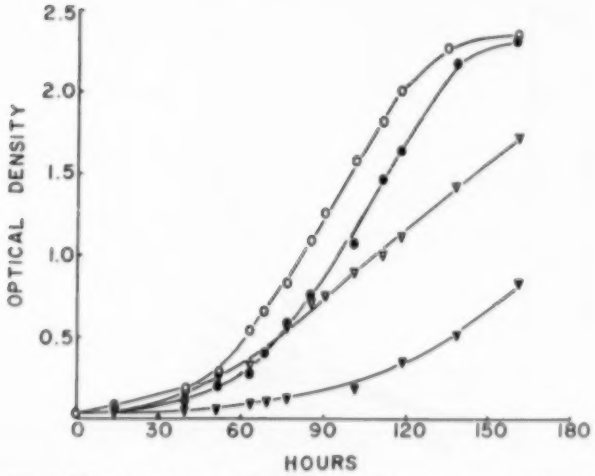

FIG. 1. Growth of *Mycobacterium tuberculosis* in liquid media on the shaker. Curves ○—○ and ●—● are for strain 198ARB grown in media with and without serum albumin, respectively; curves ▽—▽ and ▼—▼ are for strain H37Rv grown in media with and without serum albumin, respectively.

monium citrate, 0.05 gm. $MgSO_4 \cdot 7H_2O$, 0.15 gm. sodium citrate, 0.0005 gm. $CaCl_2$, 0.001 gm. $ZnSO_4$ and 0.0001 gm. $CuSO_4$. When serum albumin was desired in the medium, the above constituents were dissolved in 900 ml. of distilled water and autoclaved, after the medium cooled, 100 ml. of sterile (sintered glass filtered) 5 per cent fraction V of bovine plasma[3] were added.

Optical densities were read with a Coleman nephocolorimeter (model 9) at 430 mμ in 250 or 500 ml. side-arm flasks until an optical density near 0.500 was reached. The turbidity reading is directly proportional to the concentration of bacilli until an optical density of approximately 0.500 is obtained. Cultures more dense than this were diluted with the original Tween® medium (or the Tween®-albumin medium) before optical density determinations were made. To keep growth dispersed it was found necessary to add additional Tween® 80 between the fifth and the eighth days; usually 0.1 ml. of a 5 per cent solution was added per 10 ml. of medium.

The volume of medium contained in each 250 ml. side-arm Erlenmeyer flask usually was 40 ml. The inoculum was 0.6 to 0.8 ml. of a six- or seven-day-old culture which gave in most experiments an optical density of approximately 0.030 immediately after inoculation. Flasks were placed upon a reciprocating shaker[4] making 50 excursions per minute with a 4-inch stroke. The growths in these flasks were measured turbidimetrically and compared with the values obtained with stationary flasks. Although no viable counts are presented, Fenner and Leach (11, 12) and Smith (13) have shown that there is a good correlation between total viable units or particles and turbidimetric data.

RESULTS

In figure 1 the nephelometric readings are plotted arithmetically. The resulting curves show that the yield of bacilli with strain H37Rv in terms of bacterial

[3] Fraction V of bovine plasma is manufactured by Armour Laboratories, Chicago, Illinois.

[4] Tier-type shaker is manufactured by the New Brunswick Scientific Co., New Brunswick, New Jersey.

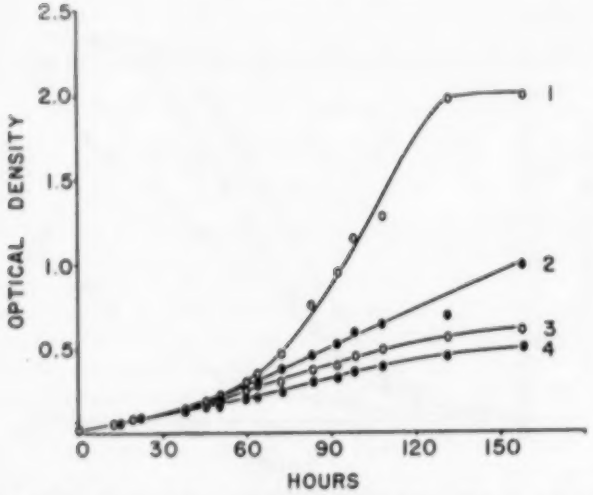

FIG. 2. Effect of shaking on growth of *Mycobacterium tuberculosis*. Curves 1 and 2 are for strains 198ARB and H37Rv, respectively, grown on the shaker; curves 3 and 4 are for strains 198ARB and H37Rv, respectively, grown stationary. All cultures were grown in a medium without serum albumin.

mass (optical density) was much less than that for 198ARB. In every case the growth of the strains employed was slower in the medium which lacked serum albumin. It may be seen from these curves that, under conditions of agitation, nearly maximum growth was obtained in approximately six days with strain 198ARB. In contrast, with strain H37Rv, maximum growth was not obtained at a comparable time even though the optical density was approximately 1.7. Maximum growth with strain H37Rv usually was not obtained until after seven to ten days.

The results of one of many experiments designed to demonstrate the effect of shaking growing cultures are contained in figure 2. Although the graphs presented in figure 2 represent the growth of the strains in the Tween® liquid medium without serum albumin, comparable results have been obtained with stationary cultures in the Tween®-albumin medium and compared with agitated cultures in the same medium. It was apparent that, if oxygen became a limiting factor as growth progressed in stationary cultures, faster growth would occur if the flasks were placed upon the shaker. This would be true, however, only after fairly turbid growth was obtained and there was an increased oxygen requirement. Conversely, cultures which have grown for several days and are then removed from the shaker should show a marked decrease in cell yield when compared with cultures shaken constantly and grown for the same length of time. That this does take place is indicated by the break in the growth curve in figure 3 obtained when H37Rv was removed from the shaker and kept stationary (except for the brief shaking required to resuspend the growth for turbidity measurements).

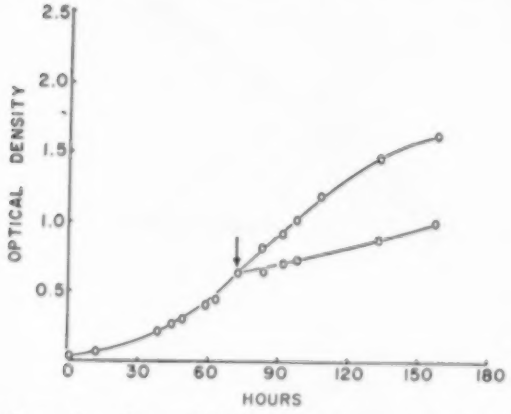

FIG. 3. Effect of removing cultures of *Mycobacterium tuberculosis* H37Rv from the shaker. Arrow indicates time at which replicate flask was removed from the shaker and held stationary at 37°C.

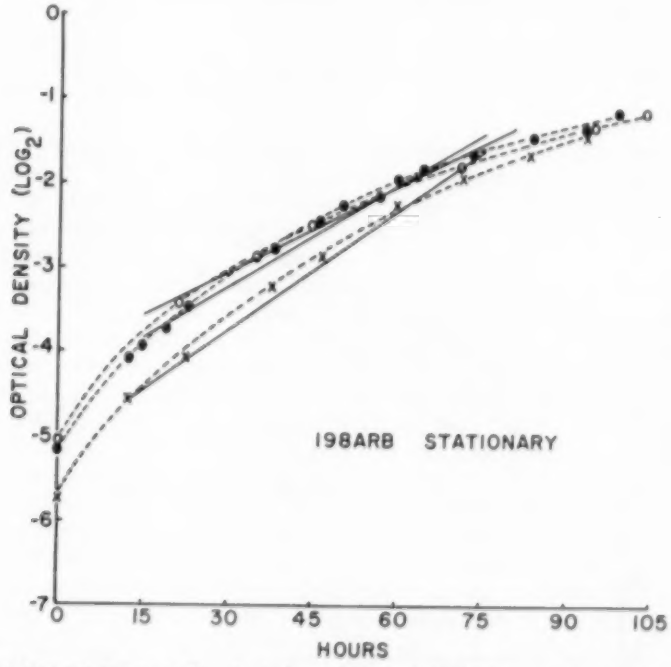

FIG. 4. Growth curves of *Mycobacterium tuberculosis* 198ARB in stationary cultures in a medium without serum albumin.

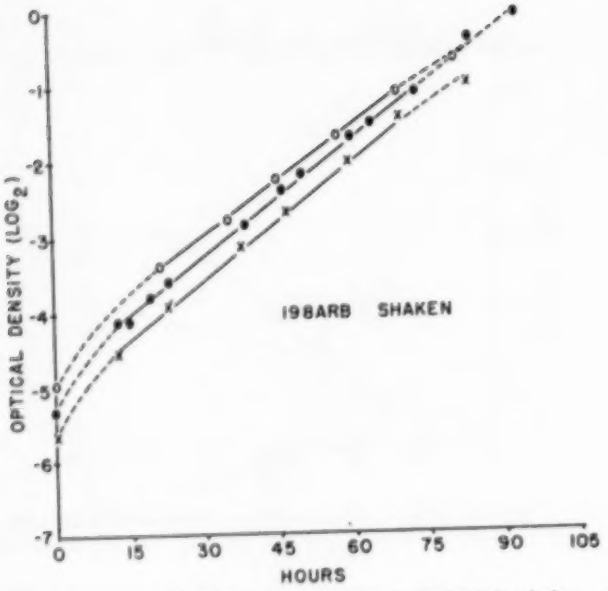

FIG. 5. Growth curves of *Mycobacterium tuberculosis* 198ARB in shaken cultures in a medium without serum albumin.

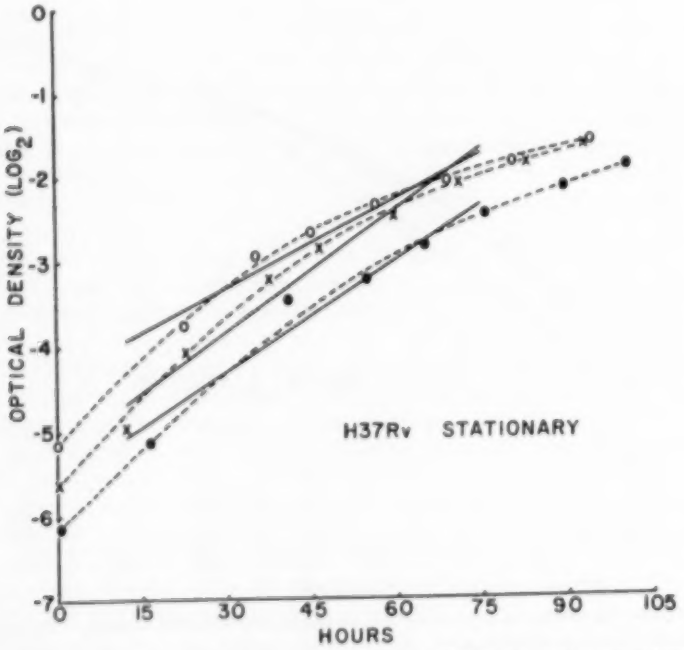

FIG. 6. Growth curves of *Mycobacterium tuberculosis* H37Rv in stationary cultures in a medium containing serum albumin.

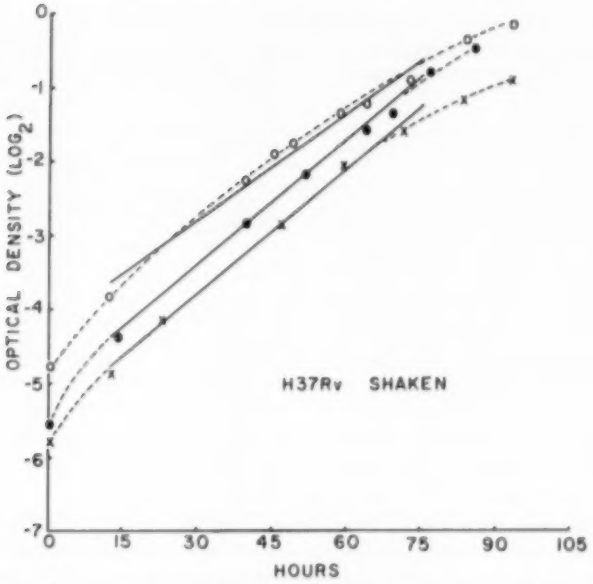

FIG. 7. Growth curves of *Mycobacterium tuberculosis* H37Rv in shaken cultures in a medium containing serum albumin.

Moreover, several experiments have been performed in which three- to five-day-old stationary cultures have been placed upon the shaker; much faster growth was observed in these flasks than in others in the same experiment which were kept stationary. Similar results have recently been reported by Halpern and Kirchheimer, using aerated cultures (14).

The exponential growth rates of the strains grown in Tween® medium are obtained from figures 4 to 7 in which the $\log_2$ of the optical densities are shown graphically. Optical densities of 0.33 to 0.54, equivalent to -1.60 to -0.89 $\log_2$ units, were obtained in approximately 72 hours with the shaken cultures of *M. tuberculosis*. The generation times calculated from the curves for the strains 198ARB and H37Rv grown in shaken cultures in the Tween®-albumin medium were 17.8 hours and 17.9 hours, respectively. The generation times were calculated from the straight line fitted to the data, using the method of least squares in a manner employed by Fenner and Leach (11). Optical densities obtained between 10 and 76 hours were used for these calculations; it is during this interval that logarithmic growth is manifested by *M. tuberculosis* (11, 12).

It is interesting to note that the lines drawn for shaken cultures fit the data very well, whereas straight lines for the stationary cultures can only be drawn with the aid of mathematical treatment. If the data are examined, it will be noted that the stationary cultures give a smooth curve rather than a straight line characteristic of logarithmic growth. As shown in table 1, the generation times

TABLE 1

GENERATION TIMES OF MYCOBACTERIUM TUBERCULOSIS IN SHAKEN AND STATIONARY CULTURES IN TWEEN® MEDIUM WITH AND WITHOUT SERUM ALBUMIN

Medium	Strain 198ARB		Strain H37Rv	
	Shaken	Stationary	Shaken	Stationary
	hours	*hours*	*hours*	*hours*
With serum albumin	18.4	29.4	14.8	18.6
	17.2	21.2	17.9	20.8
	17.5		17.9	23.2
			20.8	
Average	17.8	25.3	17.9	20.5
Without serum albumin	20.8	21.2	19.6	24.4
	18.9	23.8	22.2	26.3
	19.2	29.4	21.2	
Average	19.6	24.8	21.0	25.4

of shaken cultures were shorter than those obtained with stationary cultures. A beneficial effect of serum albumin in the growth medium is also shown in table 1; this effect was demonstrated with strain H37Rv in both shaken and stationary cultures, but only in shaken cultures with strain 198ARB.

DISCUSSION

In the experience of the present writers, logarithmic growth of *M. tuberculosis* has been obtained in liquid culture media only when the cultures are growing under optimal conditions, that is, when the cultures are agitated by shaking or stirring. This is indicated by the curves and the straight lines fitted to the curves in figures 4 to 7. There is an increasing oxygen requirement that accompanies the increase in turbidity, and this requirement can most easily be satisfied by agitation. Although logarithmic growth may occur only within the first three or four days after inoculation of liquid media, shaken cultures will continue to grow at a more rapid rate than stationary cultures so that a given amount of growth is obtained at a much earlier time with agitated cultures. From an examination of the data obtained with stationary cultures, it appears that logarithmic growth is not obtained under the conditions of these experiments with 198ARB or H37Rv; the data plotted using $\log_2$ do not give a straight line characteristic of logarithmic growth. If the proper conditions could be provided, *M. tuberculosis* would grow in a logarithmic manner to nearly maximum turbidity in a manner similar to other aerobic microorganisms.

Two papers on the arithmetic linear growth of the tubercle bacillus were written by Fisher and co-workers (15, 16) and an explanation for this arithmetic growth was made by Volk and Myrvik (17), using typical aerobes and a BCG strain of *M. tuberculosis*. They reported that the rate of growth in stationary cultures is a function of the diffusion of oxygen into the medium; logarithmic

growth curves were obtained with shaken cultures. Subsequently, Halpern and Kirchheimer (14, 18) reported "that mycobacteria inoculated into a culture medium which subsequently is aerated or oxygenated continuously grow logarithmically over a prolonged period of time, without the occurrence of an arithmetically linear growth phase." The present results with virulent *M. tuberculosis* extend these recent reports.

An explanation can be given for the unfavorable results reported in the literature with agitated cultures of *M. tuberculosis*. It may be simply that there is an optimum rate of agitation for the size of the inoculum, volume and surface area of the medium, shape of the culture vessel, constituents and surface tension of the medium, and similar factors, and that some investigators used a rate of agitation that was much too fast. It is conceivable that a vigorous agitation of suspensions of *M. tuberculosis* could cause the cells to aggregate so that growth and cell division would not reflect actual cell numbers. It has been the present writers' experience that old (more than two weeks) cultures, freshly inoculated liquid cultures from slants, and, in some instances, vigorously agitated cultures are not well dispersed. It may be that a certain amount of "free space" between cells in a liquid culture is required to provide a nonaggregated suspension. That this may be true is indicated by the difficulty one experiences in resuspending cells after they have been centrifuged. The nature of the fats and waxes of the cell undoubtedly influence the manner of growth of *M. tuberculosis*.

It appears from the results reported here that the serum albumin stimulates the rate of growth whether the cultures are shaken or held stationary. Although these experiments do not differentiate between a possible growth factor activity and the detoxifying action of serum albumin, experiments in progress using a continuous culture system may provide some objective data to resolve this question. Other experiments on the utilization of partially hydrolyzed proteins and serum albumin as medium constituents may indicate whether or not a growth-stimulating activity is present in large amino acid complexes.

SUMMARY

A study has been made of the effects of agitation on growing cultures of *Mycobacterium tuberculosis*. Contrary to some reports in the literature, growth of the organism is more rapid and greater cell numbers are obtained in shaken than in stationary cultures. Generation times obtained in a serum albumin-Tween® medium with strain 198ARB were 17.8 and 25.3 hours, respectively, for cultures shaken and stationary; comparable results obtained with strain H37Rv were 17.9 and 20.5 hours. Generation times obtained with strain 198ARB in a Tween® medium without serum albumin were 19.6 and 24.8 hours, respectively, for cultures shaken and stationary; comparable results obtained with strain H37Rv were 21.0 and 25.4 hours.

SUMARIO

Crecimiento del Mycobacterium Tuberculosis en Medios Líquidos

Este estudio trata de los efectos de la agitación sobre los cultivos en desarrollo del *Mycobacterium tuberculosis*. En contraposición a algunos informes en la literatura, se obtiene

la proliferación del microbio en forma más rápida y en mayores cantidades de células en los cultivos agitados que en los dejados en reposo. Los tiempos de generación obtenidos en un medio de seroalbúmina-Tween® con cepas 198ARB fueron de 17.8 y 25.3 horas, respectivamente, con cultivos agitados y dejados en reposo; resultados comparables con la cepa H37Rv fueron 17.9 y 20.5 horas. Los tiempos de generación obtenidos con la cepa 198ARB en un medio de Tween® sin seroalbúmina fueron de 19.6 y 24.8 horas, respectivamente, para cultivos agitados y en reposo; resultados comparables con la cepa H37Rv fueron 21.0 y 25.4 horas.

RESUME

Croissance du Mycobactererium tuberculosis dans les milieux liquides

Les effets de l'agitation sur les cultures du *Mycobacterium tuberculosis* ont été étudiés. Contrairement à certains rapports publiés, on obtient une croissance plus rapide du germe et un nombre de cellules plus grand dans les cultures que l'on agite que dans les cultures immobiles. Les temps de reproduction obtenus dans le milieu sérum albumine Tween® avec les souches 198ARB étaient 17.8 et 25.3 respectivement, pour les cultures agitées et pour les cultures immobiles; les résultats comparables qui ont été obtenus avec la souche H37Rv étaient, respectivement, 17.9 et 20.5 heures. Les temps de reproduction obtenus avec la souche 198ARB dans un milieu de Tween® sans sérum-albumine étaient 19.6 et 24.8, respectivement, pour les cultures agitées et pour les cultures immobiles; les résultats comparables obtenus avec la souche H37Rv étaient 21.0 et 25.4 heures.

Acknowledgment

The writers are indebted to Dr. Byron Olson for his stimulating discussions and advice.

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THE RELATIONSHIP BETWEEN THE CATALASE ACTIVITY, HYDROGEN PEROXIDE SENSITIVITY, AND ISONIAZID RESISTANCE OF MYCOBACTERIA[1]

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INTRODUCTION

Since the discovery (1) that in small concentrations isoniazid inhibits the growth of mycobacteria *in vitro*, evidence has been accumulating to suggest that this action is due in some way to the "severe restriction of porphyrin-containing enzymes" (2). In 1954, Middlebrook (3) reported that colonies of isoniazid-resistant strains of *M. tuberculosis* H37Rv, growing on a solid oleic-acid–albumin–agar medium, were unable to break down hydrogen peroxide. Similar results were reported by Peizer and Widelock (4), who later, however, found (5) no one significant concentration of isoniazid at which cultures of tubercle bacilli from patients became catalase inactive. From these observations, it seemed probable that the action of isoniazid involved inhibition, either of catalase synthesis or of the activity of the formed enzyme. Bacterial cells which are deficient in catalase might be expected to show greater susceptibility to hydrogen peroxide, and some evidence was produced to support this view (6).

Since 1952 this Department has carried out the bacteriologic investigations in a chemotherapeutic trial with patients suffering from chronic pulmonary fibrocaseous tuberculosis (7–10). Among the practical problems which arose during the course of this work has been the interesting one of the difficulty experienced in culturing *M. tuberculosis* from some specimens of sputum which were microscopically positive when stained by the Ziehl-Neelsen method. The patients from whom such specimens were obtained had usually received treatment with isoniazid, either alone or together with other drugs, for six to eight weeks. After a short period, the infection in these patients either relapsed so that their sputum was again frankly positive by both techniques or responded to treatment so that the sputum became negative for tubercle bacilli both microscopically and on culture.

The discrepancy between the results obtained by cultural and microscopic techniques might be explained by the emergence, at that stage in treatment, of small numbers of drug-resistant organisms. Before culture, the sputum specimens were concentrated by the method of Jungmann and Gruschka (11) which involved the use of hydrogen peroxide. If isoniazid-resistant cells were especially susceptible to the killing action of hydrogen peroxide, they might fail to survive sputum concentration in sufficient numbers to yield a positive culture.

Experiments were therefore performed to investigate further the relationship

[1] From the Department of Bacteriology, Guy's Hospital Medical School, London, England.

[2] In receipt of a grant from the Guy's Hospital Endowments Fund.

TABLE 1

DESIGNATION, SOURCE, QUANTITATIVE DRUG SUSCEPTIBILITIES, AND CATALASE ACTIVITY OF STRAINS USED

Designation	Strain	Source	Drug Susceptibilities γ *per ml.*			Catalase Activity (Middlebrook Method)
			Isoniazid	Streptomycin	PAS	
A	*M. tuberculosis* var. *hominis* H37Rv	Dr. R. J. W. Rees, Mill Hill	0.1	8.00	10.00	+
B	*M. tuberculosis* var. *hominis*	Laboratory trained	200.0	8.00	1.00	−
C	*M. tuberculosis* var. *hominis*	Laboratory trained	1.0	400.00	10.00	+
D	*M. tuberculosis* var. *hominis*	Laboratory trained	100.0	20.00	10.00	−
E	*M. tuberculosis* var. *hominis*	Patient's strain	2.0	400.00	20.00	+
F	*M. tuberculosis* var. *hominis*	Patient's strain	100.0	10.00	10.00	−
G	*M. tuberculosis* var. *hominis*	Patient's strain	2.0	10.00	10.00	+
H	*M. tuberculosis* var. *hominis*	Patient's strain	8.0	10.00	1.00	+
I	*M. tuberculosis* var. *hominis*	Patient's strain	1.0	8.00	10.00	+
J	*M. tuberculosis* var. *bovis* BCG	Dr. R. J. W. Rees, Mill Hill	0.1	0.64	10.00	+
K	*M. tuberculosis* var. *bovis* BCG	Laboratory trained	10.0	0.64	10.00	−
L	*Staph. aureus* Oxford	NCTC 6571	1,500	0.03	1,000	+

between isoniazid resistance, catalase production, and hydrogen peroxide sensitivity in mycobacteria.

MATERIALS AND METHODS

Cultures and media: In table 1 are shown the designation and source of the cultures used, and their quantitative susceptibilities to isoniazid,[3] streptomycin, and para-aminosalicylic acid (PAS). Those strains, originally isolated from patients' sputum (*E, F, G, H,* and *I*), were obtained by culturing the sputum, after concentration by Jungmann's method, on Löwenstein-Jensen slopes, each containing 4 ml. of slanted medium.[4] The growth was subcultured after six weeks' incubation into liquid Dubos-albumin medium (12).

All strains of mycobacteria were maintained in liquid Dubos-albumin medium at 37°C. and subcultured at fortnightly intervals. Drug susceptibility tests were performed on Löwenstein-Jensen medium.

The Oxford strain of *Staphylococcus aureus* was maintained on nutrient agar.

Two media were prepared for growing cells for use in the Warburg apparatus:

1. *Fumarate medium.* This was a modification of the Dubos medium and contained KH_2PO_4, 1 gm.; $Na_2HPO_4 \cdot 12H_2O$, 6.5 gm.; $MgSO_4 \cdot 7H_2O$, 0.4 gm.; 20 per cent casein, hydrolysate, 10 ml.; 10 per cent w/v Tween® 80, 5 ml.; sodium fumarate, 1.7 gm. per liter of medium. The pH was adjusted to 6.8 and the medium was sterilized by autoclaving.
2. *Digest broth.* Hartley modification.

Susceptibility tests to hydrogen peroxide: These were carried out on Löwenstein-Jensen slopes containing falling concentrations of hydrogen peroxide. Ten doubling dilutions in

[3] Isoniazid (Pycazide®) was kindly supplied by Smith and Nephew Research, Ltd., Welwyn Garden City, Hertfordshire, England.

[4] Supplied by the Southern Group Laboratory, Park Hospital, Hither Green, London, S. E. 13, England.

saline of 6 per cent w/v aqueous hydrogen peroxide 20 volume were freshly prepared, and 6.04 ml. of each dilution was added to a Löwenstein-Jensen slope, using a standard dropping pipette (13, 14) calibrated to deliver 0.02 ml. The final concentrations in the slopes ranged from 0.06 per cent to 0.00005 per cent. After resting the slopes for half an hour to allow even diffusion, they were inoculated with 0.02 ml. of a ten-day-old Dubos culture, incubated at 37°C., and read weekly for six weeks.

The killing effect of hydrogen peroxide: In order to obtain information on the killing effect of hydrogen peroxide, liquid Dubos cultures of strains *A* to *I* were treated with hydrogen peroxide at room temperature for five minutes, washed to remove excess hydrogen peroxide, and were then inoculated onto Löwenstein-Jensen slopes. Viable counts were performed before and after the treatment. The nine Dubos cultures used ranged from eight to twelve days old. One-tenth of a milliliter of each culture was treated with 2 ml. of 2 per cent hydrogen peroxide for five minutes in sterile conical tubes. These were then centrifuged for five minutes at 3,000 r.p.m. and the supernatant fluid was removed. Two milliliters of sterile normal saline were added and the tubes were shaken. They were then centrifuged for a further five minutes at 3,000 r.p.m., after which the supernatant liquid was removed and a viable count was performed on the deposit.

To determine the numbers of surviving cells after periods of treatment longer than five minutes, a further experiment was carried out with one-week-old Dubos cultures of strains *A*, *B*, and *C*. One milliliter of each culture was treated with 1 ml. of 1 per cent hydrogen peroxide. After intervals of five, fifteen, twenty-five, thirty, thirty-five, and forty minutes, each tube was shaken and a sample was removed. Normal saline was added to the sample and, after centrifuging, the supernatant fluid was discarded and a viable count was performed on the deposit and on the original inoculum.

Viable counts: These were carried out by making serial transfers of the deposit or culture to give dilutions of 10^{-2}, 10^{-4}, and 10^{-6}. These were then inoculated onto Löwenstein-Jensen slopes, and the number of separate colonies growing on each slope was recorded after six weeks' incubation. The one-hundred fold dilutions were made by adding 0.02 ml. from a standard pipette to 2.0 ml. of normal saline. Fresh pipettes were used for each transfer and for mixing.

Measurement of catalase activity: Catalase activity was detected by two methods:

1. *Middlebrook method* (3)
   A mixture of hydrogen peroxide and Tween® 80 was added to the colonies on the surface of a Löwenstein-Jensen slope. A positive reaction consisted of the active evolution of bubbles of gas from the submerged colonies within thirty seconds of the application of the test solution.
2. *Warburg method*
   The method of Clark (15) was used. Hydrogen peroxide was added from the side arm of the Warburg apparatus to the catalase-containing preparation and the activity was measured in terms of the volume of oxygen evolved.

EXPERIMENTAL RESULTS

Catalase Activity of Isoniazid-Susceptible and Isoniazid-Resistant Strains of Mycobacteria

The nine strains of *M. tuberculosis* var. *hominis* (*A* to *I*) and two strains of BCG (*J* and *K*) were grown on Löwenstein-Jensen slopes and their catalase activity was tested by the Middlebrook method. As shown in table 1, strains showing a high degree of isoniazid resistance produced no detectable catalase, whereas catalase was found in strains which were susceptible or only moderately resistant to isoniazid.

The catalase activity of BCG strains *J* and *K* was tested in the Warburg

TABLE 2

EFFECT OF ISONIAZID ON CATALASE ACTIVITY AS MEASURED IN THE WARBURG APPARATUS

Source of Catalase	Growth Medium	µl. of Oxygen Evolved per Minute	Effect of Isoniazid on Oxygen Evolved
Strain *J*	Fumarate	240 per mg. dry weight	None
Strain K	Fumarate	0	—
Strain K	Fumarate + 10 γ of hemin per ml.	0	—
Blood (1:10 dilution)	—	420 per ml.	None
Crystalline catalase (10^{-6} dilution)	—	250 per ml.	None
Hemin (100γ per ml.)	—	320 per ml.	None
Strain L	Digest broth	680 per mg. dry weight	None
Strain L	Digest broth + 25 mg. of isoniazid per ml.	710 per mg. dry weight	None

apparatus, with the results shown in table 2. The drug-susceptible strain *J* decomposed hydrogen peroxide with the liberation of oxygen, whereas the resistant strain *K* failed to do so. It is known that some isoniazid-resistant strains of mycobacteria require hemin for growth under certain conditions (16, 17). Since catalase is a porphyrin-containing enzyme and may be synthesized from hemin, an attempt was made to produce catalase activity in an isoniazid-resistant strain by adding hemin to the growth medium. With this strain, growth occurred in the absence of hemin and was stimulated by hemin, but the presence of catalase could not be detected.

The effect of isoniazid on catalase activity: The effect of isoniazid on the decomposition of hydrogen peroxide by catalase from nonbacterial sources was measured by adding hydrogen peroxide to the catalase preparation in the Warburg flasks, with and without the addition of 10^{-4} molar isoniazid. The catalase activities of blood, hemin, and a crystalline catalase preparation[5] were all found to be unchanged by isoniazid (table 2). The catalase activities of an isoniazid-susceptible strain of BCG (*J*) and of *Staphylococcus aureus* (*L*) were found to be unaffected by isoniazid. The micrococci were also grown in the presence of subinhibitory concentrations of isoniazid (25 mg. per ml.) in order to determine whether isoniazid could interfere with the synthesis of catalase under these conditions. The organisms remained strongly catalase-positive and unaffected by isoniazid (table 2).

Hydrogen peroxide susceptibility of strains A to I: In figure 1 are shown the highest concentrations of hydrogen peroxide, in Löwenstein-Jensen slopes, on which growth of these strains occurred after six weeks' incubation. The initial inoculum size of each strain was estimated by the method of viable counting in order to detect gross differences between the strains which might, in them-

[5] Supplied by Worthington Biochemical Corporation, Freehold, New Jersey.

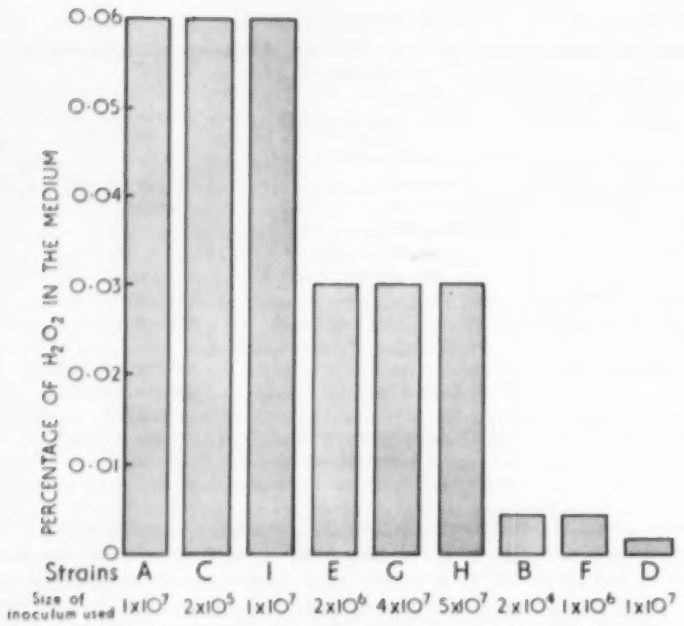

FIG. 1. The susceptibility of strains to hydrogen peroxide on Löwenstein-Jensen slopes.

selves, account for variations in apparent susceptibility. It is clear from the figures obtained that variations in inoculum size do not account for these differences. It may be seen that three isoniazid-susceptible strains (*A*, *C*, and *I*) grew in the highest concentration of hydrogen peroxide used (0.06 per cent). The end-point for three strains of intermediate susceptibility (*E*, *G*, and *H*) was 0.03 per cent; for two resistant strains (*B* and *F*), 0.00375 per cent; and for one isoniazid-resistant strain (*D*), 0.0018 per cent hydrogen peroxide.

Killing effect of hydrogen peroxide:

After five minutes' exposure:

In table 3 are shown the number of surviving cells of the nine strains of *M. tuberculosis* var. *hominis* (*A* to *I*) after exposure to 2 per cent hydrogen peroxide for five minutes. Although a certain proportion of all strains were killed, it was only in the case of the strains susceptible to isoniazid that any growth occurred after this treatment.

Effect of longer periods of exposure:

Three strains, *A*, *B*, and *C* were exposed to 0.5 per cent hydrogen peroxide for forty minutes and sampled at intervals. The results obtained are shown in figure 2. The isoniazid-resistant strain *B* was completely killed after five minutes' exposure, whereas some cells from the other strains (*A* and *C*) appeared to be able to withstand treatment with hydrogen peroxide for forty minutes at least.

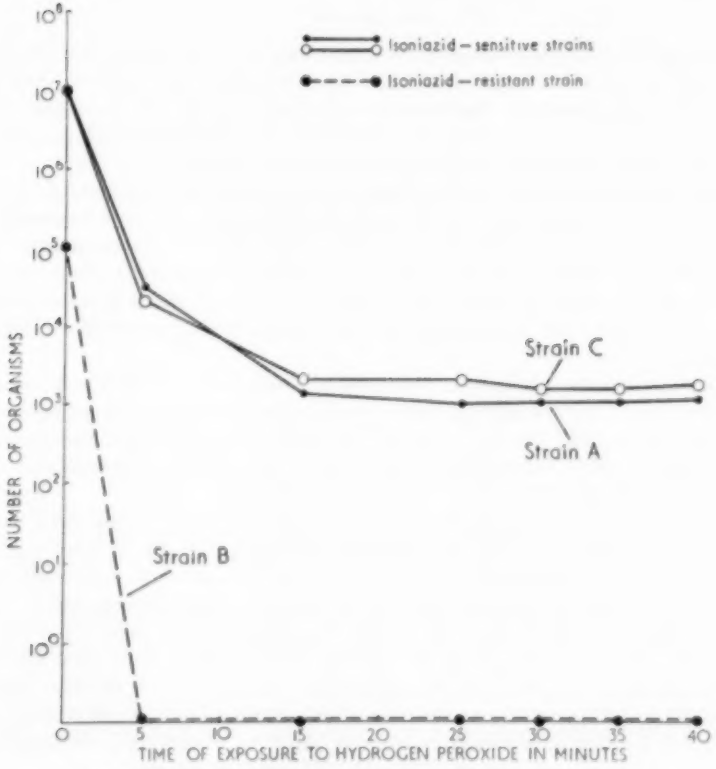

FIG. 2. The rate of killing by 0.5 per cent hydrogen peroxide.

TABLE 3

THE EFFECT OF FIVE MINUTES' EXPOSURE TO 2.0 PER CENT HYDROGEN PEROXIDE

Strain	Viable Count* Before Treatment	Viable Count* After Treatment
A	2×10^7	6×10^5
B	6×10^8	0
C	10^7	8×10^4
D	10^8	0
E	6×10^5	8
F	10^7	0
G	4×10^5	3×10^5
H	2×10^5	10^4
I	10^5	30

* Number of viable organisms per ml.

DISCUSSION

The experiments described provide confirmation of Middlebrook's observation that isoniazid-resistant strains of mycobacteria have reduced catalase activity. In addition they show that this deficiency is specific to isoniazid resistance, since the strains which are resistant to streptomycin (*C*, *E*) and to PAS (*E*) retain the ability to decompose hydrogen peroxide.

The experiments in the Warburg apparatus indicate that the activity of preformed catalase is unchanged by the presence of isoniazid, whether the source of catalase is bacterial or not. It seems probable, therefore, that the relation between isoniazid and catalase involves the synthesis of the enzyme rather than its activity. As suggested by Knox (17), it may not be the synthesis of catalase itself which is inhibited, but the synthesis of other peroxidases, possibly those specific to mycobacteria.

There is no information as to the level of drug resistance at which catalase activity is lost, nor whether the loss occurs suddenly or gradually as resistance increases. The three strains (*E*, *G*, *H*) of intermediate isoniazid susceptibility also appear to show intermediate levels of hydrogen peroxide susceptibility, but this may not be significant.

The metabolic loss of catalase in isoniazid-resistant strains is associated with a corresponding increase in susceptibility to hydrogen peroxide. A solid medium was used in the experiments to demonstrate this because of the ease with which the amount of growth could be observed. Hydrogen peroxide decomposes rapidly on this, and probably on all, media. The concentration of hydrogen peroxide in each slope should not, therefore, be regarded as an absolute value, and different end-points would be obtained with methods involving the use of other media. The difference between the hydrogen peroxide susceptibility levels of isoniazid-susceptible and isoniazid-resistant strains is, however, considered to be significant and may be correlated with the difference in catalase activity.

The experiments involving exposure to hydrogen peroxide for various periods show that all isoniazid-resistant cells were killed after five minutes' treatment. On the other hand, the isoniazid-susceptible strains were considerably more resistant to treatment with hydrogen peroxide.

All efficient methods of concentrating sputum involve the killing of a proportion of the mycobacteria present, although the extent to which bacterial cells are protected by mucus and other factors is not known. It is clear that a special danger may exist in the use of Jungmann's method of concentrating sputum. In this method, sputum is treated with approximately 0.75 per cent hydrogen peroxide for thirty minutes. If the mycobacteria are present only in small numbers and are isoniazid-resistant, from the present observations it appears likely that none would survive. If the population were larger and contained both isoniazid-susceptible and isoniazid-resistant cells, then a selective killing action might be expected, and the results of subsequent drug-susceptibility tests would not give a true indication of the type of organism originally present in the sputum.

SUMMARY

The observation that some isoniazid-resistant strains of mycobacteria are deficient in catalase was confirmed. This deficiency was specific to isoniazid resistance and was associated with an increase in susceptibility to the lethal effect of hydrogen peroxide.

The activity of a number of catalase preparations was found to be unaffected by isoniazid, and it is suggested that isoniazid interferes with catalase or peroxidase synthesis in mycobacteria.

It is proposed that methods of sputum concentration involving the use of hydrogen peroxide should be employed with caution, since they may exert a selective killing action on mixed populations of mycobacteria containing both isoniazid-susceptible and isoniazid-resistant organisms.

SUMARIO

La Relación entre la Actividad Catalásica, la Susceptibilidad al Peróxido de Hidrógeno y la Resistencia a la Isoniacida de las Micobacterias

Confírmose la observación de que algunas cepas isoniacidorresistentes de micobacterias son deficientes en catalasa. Esta deficiencia fué específica para la resistencia a la isoniacida y estaba unida a hipersusceptibilidad al efecto letal del peróxido de hidrógeno.

La actividad de varias preparaciones de catalasa se mostró inafectada por la isoniacida, y se sugiere que la isoniacida obstruye la síntesis de la catalasa o la peroxidasa en las micobacterias.

Propónese que se empleen con cautela métodos de concentración del esputo que requieran el uso de peróxido de hidrógeno, dado que pueden ejercer una acción letal selectiva sobre poblaciones mixtas de micobacterias que contengan microbios tanto isoniacidosusceptibles como isoniacidorresistentes.

RESUME

Relation entre l'activité de la catalase, la sensibilité au peroxyde d'hydrogène et l'isoniazide-résistance des mycobactéries

L'observation d'une déficience en catalase dans certaines souches de mycobactériennes a été confirmée. Ce déficit était spécifique à l'égard de l'isoniazide-résistance et il était associé à l'accroissement de la sensibilité à l'action létale du peroxyde d'hydrogène.

Il a été découvert que l'activité d'un certain nombre de préparations de catalase n'était pas influencée par l'isoniazide et l'auteur présume que l'isoniazide s'oppose à la synthèse de la catalase ou de la peroxydase chez la mycobactérie.

L'article suggère d'effectuer avec prudence les méthodes de concentration des expectorations utilisant le peroxyde d'hydrogène, car une action létale spécifique peut s'exercer sur les populations mixtes de mycobactéries comportant des germes isoniazide-sensibles et isoniazide-résistants.

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THE NEUTRALIZATION OF ISONIAZID ACTIVITY IN MYCOBACTERIUM TUBERCULOSIS BY CERTAIN METABOLITES[1, 2]

HILDA POPE

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INTRODUCTION

Although some time has elapsed since the introduction of isoniazid as one of our most effective weapons against tuberculosis, an adequate explanation for its high and specific activity against tubercle bacilli is still lacking. A number of publications have suggested a possible mode of action for the drug, but the evidence is still insufficient to site a single locus as the primary point of attack. The present article describes some of the writer's findings on the effect of isoniazid on the growth and respiration of tubercle bacilli. Particular emphasis is placed on the ability of certain metabolites to neutralize isoniazid activity.

MATERIALS AND METHODS

The organisms employed in this study were the stock laboratory strains of tubercle bacilli, H37Rv, and its isoniazid-resistant variant, H37Rv-IR[3], resistant to greater than 10 γ of isoniazid per ml. Stock cultures of these organisms are maintained by biweekly transfer of surface growth on Proskauer-Beck synthetic medium and by weekly transfer in a Tween® 80 medium without albumin.

The basal medium used in the growth studies had a final pH of 7.0 and was of the following composition per 1,000 ml.: KH_2PO_4, 1.0 gm.; $Na_2HPO_4 \cdot 12H_2O$, 6.3 gm.; Bacto-asparagin, 2.0 gm.; Ferric NH_4 citrate, 0.05 gm.; $MgSO_4 \cdot 7H_2O$, 10 mg.; $CaCl_2$, 0.5 mg.; $ZnSO_4$, 0.1 mg.; $CuSO_4$, 0.1 mg.; Glycerol, 5 gm.; Tween® 80, 0.5 gm.

The inoculum consisted of 0.1 ml. of a seven-day Tween® 80 culture of H37Rv per 10 ml. of media. All cultures were shaken daily to resuspend the organisms and maintain a homogeneous suspension. Tests were performed in calibrated Pyrex tubes and the rate of growth was followed by taking optical density readings on a Coleman Junior spectrophotometer at 500 angstroms. A Seitz-filtered solution of isoniazid (obtained from General Biochemicals Laboratories) was added to the autoclaved culture media. The following substances were tested for their ability to antagonize isoniazid inhibition: pyridoxine, pyridoxal, pyridoxamine, niacin, nicinamide, diphosphopyridine nucleotide, biotin, thiamin, riboflavin, pantothenic acid, vitamin B_{12}, adenine, guanine, thymine, uracil, L-alanine, D-alanine, L-arginine, DL-aspartic acid, L-cystine, L-cysteine, L-glutamic acid, D-glutamic acid, glycine, L-histidine, L-leucine, DL-isoleucine, DL-norleucine, L-lysine, DL-methionine, DL-phenylalanine, L-proline, L-hydroxyproline, DL-serine, DL-threonine, L-tryptophan, L-tyrosine, DL-valine, diaminopimelic acid, sodium pyruvate, α-ketoglutarate, glutathione, cytochrome-c, yeast extract, casein hydrolysate, and nucleic acid. All labile substances and those of questionable stability were sterilized by Seitz filtration. Pyridoxal cultures were also protected from the light.

In the respiration studies, the organisms were grown on the surface of Proskauer-Beck medium for ten days. At the end of the growth period, the cells were harvested and washed

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[1] From the Department of Bacteriology, Duke University School of Medicine, Durham, North Carolina.

[2] This study was aided by a grant from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association.

[3] Obtained through the courtesy of Dr. William Steenken, Jr., Trudeau Laboratory.

twice in normal physiologic saline. They were then suspended in a small amount of sterile 0.05 M phosphate buffer, pH 6.7, and transferred to a Ten Broeck tissue grinder in order to obtain a smooth suspension of cells. The organisms were washed once in the buffer so that each milliliter of suspension contained 0.1 ml. of packed cells.

Oxygen uptake was measured in the Warburg apparatus, under air, at 37.5°C. Isoniazid was added from the side arm after a fifteen-minute equilibration period.

TABLE 1

THE EFFECT OF PYRIDOXAL ON THE INHIBITION OF H37Rv TUBERCLE BACILLI BY ISONIAZID

Concentration of Isoniazid	Concentration of Pyridoxal	O.D.* (−Log T) 14 Days
γ per ml.	*γ per ml.*	
0	0	0.289
	5	0.249
	10	0.244
	15	0.232
	20	0.216
	25	0.199
	30	0.185
	40	0.163
0.04	0	0.000
	5	0.196
	10	0.240
0.06	0	0.000
	5	0.136
	10	0.211
	15	0.234
0.08	0	0.000
	10	0.159
	15	0.199
	20	0.218
0.10	0	0.001
	10	0.074
	15	0.159
	20	0.194
	30	0.166
	40	0.123
0.15	0	0.000
	20	0.017
	25	0.147
	30	0.183
	40	0.056
0.20	0	0.000
	25	0.011
	30	0.136
	40	0.012

* O.D.—Optical density

OBSERVATIONS

Of the compounds tested for antagonism against isoniazid, activity was shown by pyridoxal, pyridoxamine, sodium pyruvate, α-ketoglutarate, guanine, nucleic acid, vitamin B_{12}, and eight of the amino acids.

Pyridoxal, employed as the hydrochloride, was the most effective of the agents tested. The relationship between the drug concentration and the concentration of the vitamin required to overcome the inhibition is shown in table 1. This relationship was more obvious at concentrations of isoniazid of 0.04 to 0.08 γ per ml. when increasing concentrations of pyridoxal were required for complete neutralization. Above this level, although an increased amount of pyridoxal was necessary for maximal antagonism, antagonism was never complete, and the higher concentrations of pyridoxal were even less effective. For example, with 0.15 γ of isoniazid per ml., maximal antagonism was obtained with 30 γ of pyridoxal per ml., but at levels of pyridoxal above this the degree of antagonism was small.

The combined effect of isoniazid and pyridoxal on tubercle bacilli resistant to isoniazid differed from the anticipated findings, as shown in table 2. Under the usual conditions of testing, this organism is resistant to 10 γ of isoniazid per ml.

TABLE 2

THE EFFECT OF PYRIDOXAL ON H37Rv TUBERCLE BACILLI RESISTANT TO ISONIAZID

Concentration of Isoniazid	Concentration of Pyridoxal	O.D.* (−Log T) 14 Days	Per Cent Inhibition
γ per ml.	*γ per ml.*		
0	0	0.357	
1		0.313	12.3
2		0.308	13.7
3		0.307	14.0
10		0.300	16.0
0	0.5	0.341	4.5
1		0.302	15.4
2		0.169	52.7
3		0.004	98.9
0	1.0	0.313	12.3
1		0.293	17.9
2		0.004	98.9
0	5.0	0.299	16.2
1		0.258	27.7
2		0.014	96.1
3		0.002	99.4
0	10.0	0.299	16.2
1		0.269	24.7
2		0.179	49.9
3		0.004	98.9

* O.D.—Optical density

When tested in the presence of noninhibitory levels of pyridoxal, however, growth was markedly inhibited. This effect could be demonstrated at concentrations as low as 0.5 γ of pyridoxal per ml. and 2.0 γ of isoniazid per ml., with maximal effectiveness at pyridoxal concentrations of 1.0 to 5.0 γ per ml.

Growth of tubercle bacilli was also inhibited by hydroxylamine, a compound known to react with aldehyde and ketone groups, but inhibition by this drug could not be overcome by including pyridoxal in the culture medium.

Pyridoxamine antagonized the activity of isoniazid, but only when minimal inhibitory concentrations of the drug were used. It was also necessary that the vitamin be sterilized by Seitz filtration since its antagonistic activity was almost completely lost when it was autoclaved with the medium.

Sodium pyruvate and α-ketoglutarate exerted a pronounced effect on isoniazid inhibition (table 3). With both of these keto acids an increase in drug concentration necessitated an increase in the concentration of antagonist to produce a comparable effect. Whereas 0.5 mg. of pyruvate per ml. produced a maximal effect with 0.05 γ of isoniazid per ml., when the drug concentration was increased to 0.5 γ per ml., this concentration of pyruvate was completely ineffective.

TABLE 3

THE EFFECT OF SODIUM PYRUVATE ON THE INHIBITION OF H37Rv TUBERCLE BACILLI BY ISONIAZID

Concentration of Isoniazid	Concentration of NaPyruvate	O.D.* (−Log T) 14 Days
γ *per ml.*	γ *per ml.*	
0	0	0.335
	50	0.330
	100	0.320
	500	0.305
	1,000	0.302
	2,500	0.299
	5,000	0.258
0.05	0	0.000
	50	0.235
	100	0.238
	500	0.251
	1,000	0.256
0.10	0	0.000
	100	0.002
	500	0.200
	1,000	0.236
	2,500	0.293
0.50	0	0.001
	2,500	0.003
	5,000	0.245

* O.D.—Optical density

TABLE 4

THE EFFECT OF PYRIDOXAL AND SODIUM PYRUVATE ON THE INHIBITION OF H37Rv TUBERCLE BACILLI BY IPRONIAZID

Concentration of Iproniazid	Concentration of Pyridoxal	O.D.* (−Log T) 14 Days	Concentration of Iproniazid	Concentration of NaPyruvate	O.D.* (−Log T) 14 Days
γ per ml.	*γ per ml.*		*γ per ml.*	*γ per ml.*	
0	0	0.322	0	0	0.312
	10	0.280		1	0.284
	25	0.270		5	0.096
4	0	0.007	4	0	0.001
	10	0.272		1	0.264
	25	0.278		5	0.094
5	0	0.006	5	0	0.002
	10	0.137		1	0.268
	25	0.273		5	0.098

* O.D.—Optical density

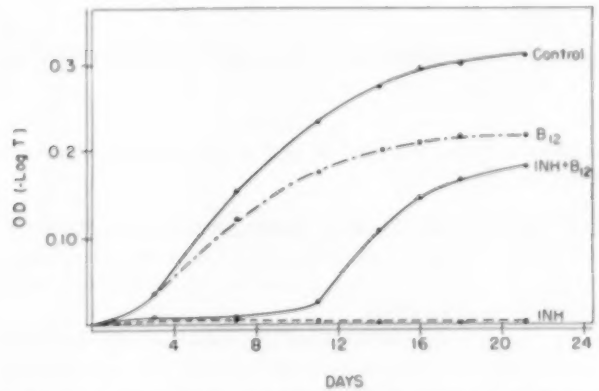

FIG. 1. The effect of vitamin B_{12} on the inhibition of growth of H37Rv tubercle bacilli by isoniazid: 0.04 γ of isoniazid (INH) per ml., 20 γ of vitamin B_{12} per ml.

Sodium pyruvate and pyridoxal also produced an antagonistic effect against iproniazid, the isopropyl derivative of isoniazid (table 4).

In figure 1 data are presented showing the effect of vitamin B_{12} on inhibition by isoniazid. At this concentration of drug (0.04 γ per ml.), inhibition was still complete after twenty-one days of incubation. In the presence of 20 γ of the vitamin per ml., growth began after a considerable lag period, but leveled off early, as did the vitamin B_{12} control. The 10 γ of vitamin B_{12} also produced a demonstrable effect, only slightly less than that produced with 20 γ. The 2 γ of vitamin B_{12} produced no effect in the presence of 0.04 γ of isoniazid per ml., but at a subinhibitory concentration of isoniazid (0.035 γ per ml.) considerable

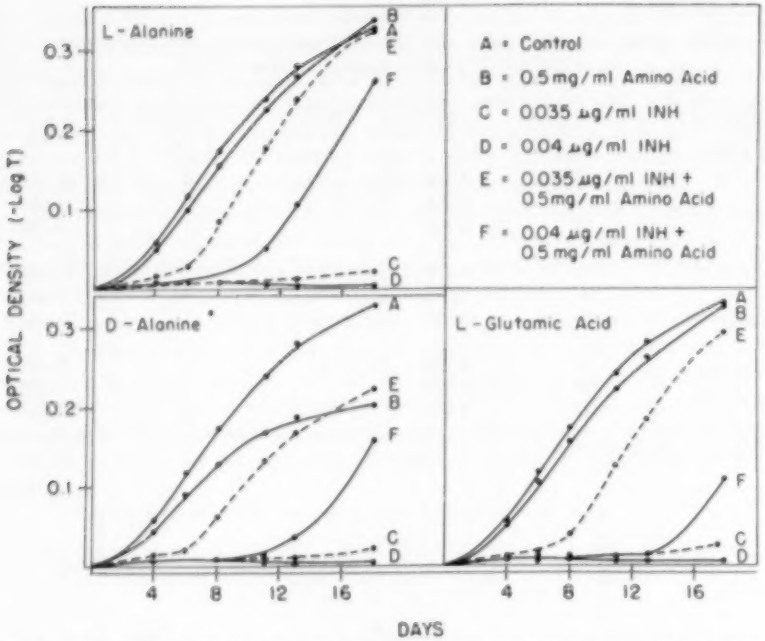

FIG. 2. The effect of alanine and glutamic acid on the inhibition of growth of tubercle bacilli by isoniazid (INH).

neutralization was observed. Maximal neutralization of 0.045 γ of isoniazid per ml. was shown by 50 γ of vitamin B_{12} per ml., but, at concentrations of 0.05 γ of isoniazid per ml. and above, concentrations up to 50 γ of vitamin B_{12} per ml. were completely without effect.

Several of the *a*-amino acids neutralized the inhibitory effect of isoniazid, but only when the drug was employed at minimal inhibitory concentrations. Even in the presence of amino acid, however, growth never quite reached the control value except with L-alanine which was the most effective of the amino acids tested (figure 2). Other amino acids which showed an antagonism for isoniazid were D-alanine, L-arginine, L-glutamic acid, D-glutamic acid, L-leucine, L-lysine, L-proline, L-tyrosine, and DL-valine (figures 3 and 4). Vitamin-free casein hydrolysate and nucleic acid (table 5) also raised the minimal inhibitory concentration of isoniazid, as did guanine (figure 5).

Respiration studies: When 1,000 γ of isoniazid were added to washed resting cells in the Warburg apparatus, there was an appreciable inhibition of their endogenous respiration. Concentrations lower than this failed to inhibit and actually caused a small stimulus to respiration.

The effect of isoniazid was also tested on the oxidation of a number of typical substrates such as glucose, glycerol, pyruvate, lactate, and acetate in order to determine, if possible, the nature of the substances whose oxidation was inhibited.

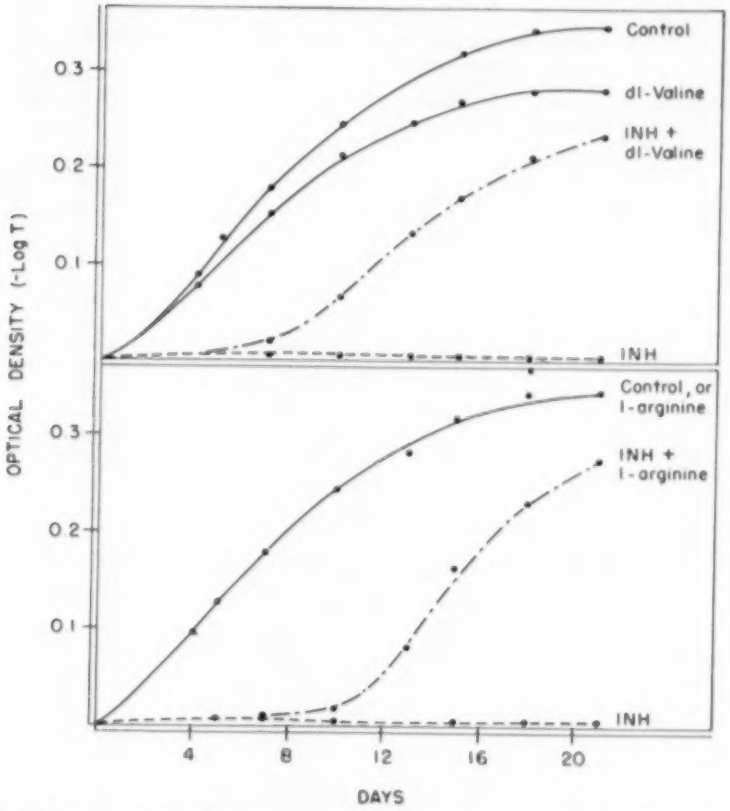

FIG. 3. The effect of DL-valine and L-arginine on inhibition of growth of H37Rv tubercle bacilli by isoniazid: 0.035 γ of isoniazid (INH) per ml.; 1.0 mg. of DL-valine per ml.; 0.5 mg. of L-arginine per ml.

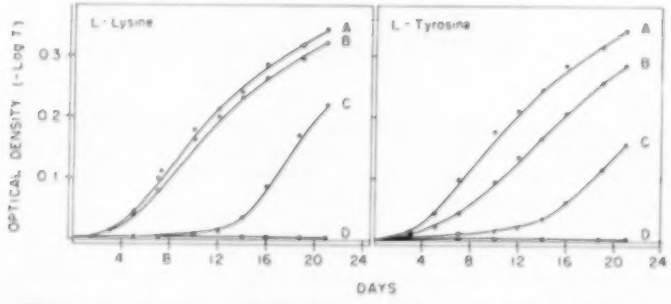

FIG. 4. The effect of L-lysine and L-tyrosine on the inhibition of H37Rv tubercle bacilli by isoniazid. *A* = control; *B* = 0.5 mg. of amino acid per ml.; *C* = amino acid plus isoniazid (INH); *D* = 0.04 γ of isoniazid (INH) per ml.

TABLE 5

THE EFFECT OF NUCLEIC ACID ON THE INHIBITION OF H37Rv TUBERCLE BACILLI BY ISONIAZID

Concentration of Isoniazid	Concentration of Nucleic Acid	O.D.* (−Log T) 15 Days
γ per ml.	*γ per ml.*	
0	0	0.294
	10	0.280
	50	0.278
	100	0.282
	200	0.248
0.04	0	0.005
	10	0.005
	50	0.221
	100	0.268
	200	0.248
0.045	0	0.004
	50	0.008
	100	0.208
	200	0.210

* O.D.—Optical density

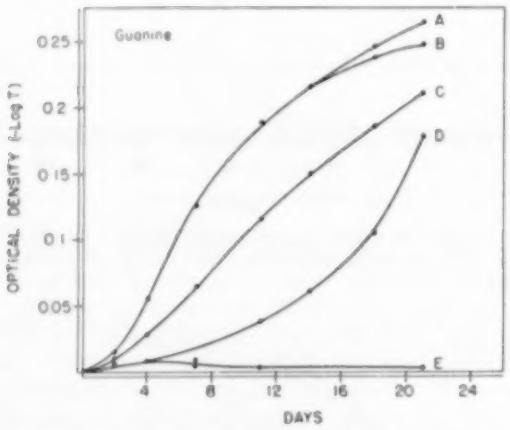

FIG. 5. The effect of guanine on the inhibition of growth of H37Rv tubercle bacilli by isoniazid (INH). *A* = control; *B* = 25 γ of guanine per ml.; *C* = 0.035 γ of isoniazid (INH) per ml. plus 25 γ of guanine per ml.; *D* = 0.04 γ of isoniazid (INH) plus 25 γ of guanine per ml.; *E* = 0.035 or 0.04 γ of isoniazid (INH) per ml.

The effect of isoniazid on pyruvate oxidation is shown in figure 6. The figures have been corrected for endogenous values. One thousand micrograms of isoniazid reduced the oxidation of 0.05 M sodium pyruvate to approximately one-

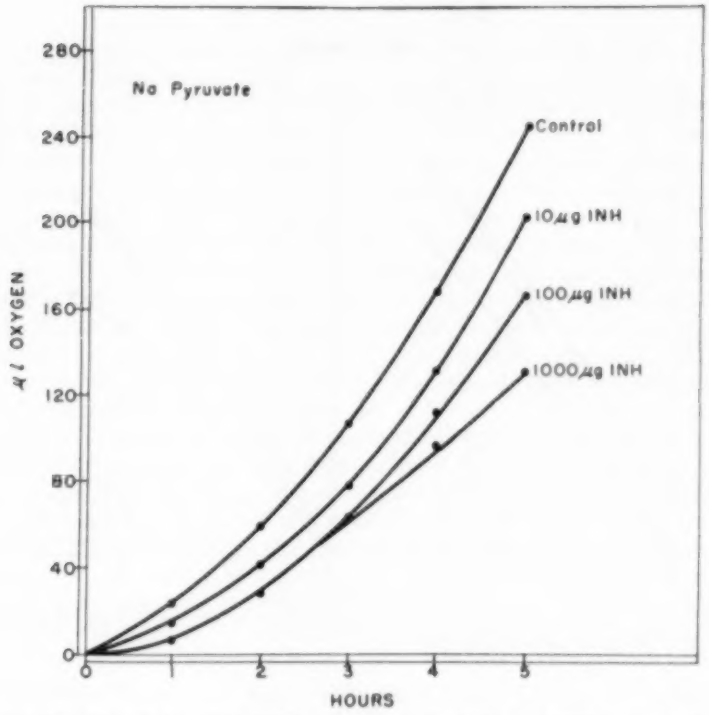

FIG. 6. The effect of isoniazid (INH) on pyruvate oxidation by H37Rv tubercle bacilli.

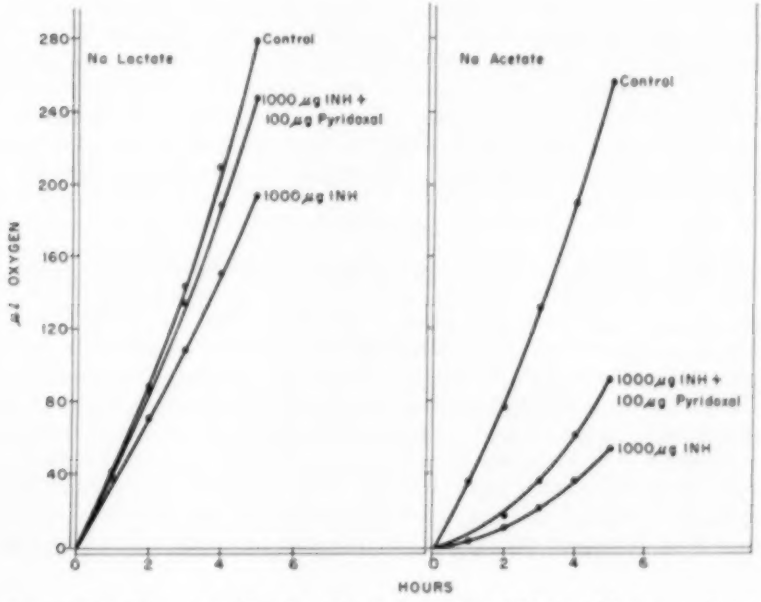

FIG. 7. The effect of pyridoxal on isoniazid (INH) inhibition of lactate and acetate oxidation in H37Rv tubercle bacilli.

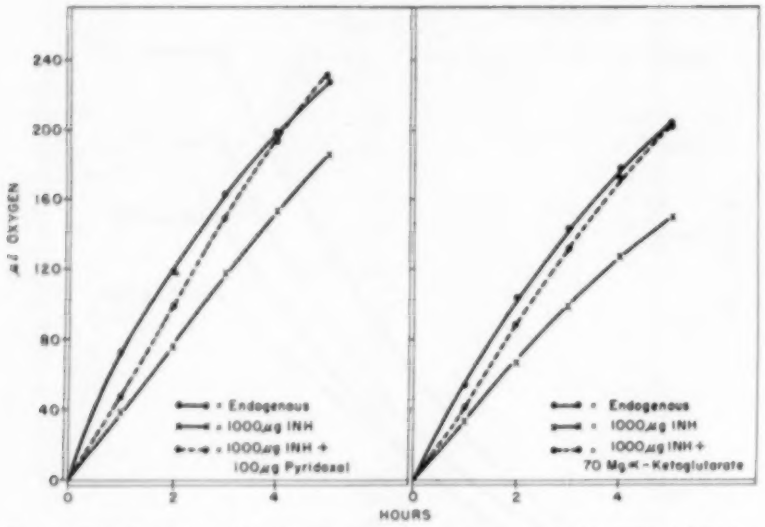

FIG. 8. The effect of pyridoxal and α-ketoglutarate on isoniazid (INH) inhibition of respiration by H37Rv tubercle bacilli.

half that of the control; 10 and 100 γ of isoniazid were also inhibitory. Similar results were obtained for other substrates tested (figure 7).

The oxidation of α-ketoglutarate was not inhibited by isoniazid. Catalytic amounts of this keto acid, however, completely neutralized isoniazid inhibition of the endogenous respiration after a five-hour incubation period, at which time the oxygen uptake approximated that of the control. Pyridoxal was also able to neutralize the inhibitory effect of isoniazid on resting cells (figure 8). Catalytic amounts of pyruvate failed to antagonize isoniazid inhibition of respiration.

The respiration of cells which had been rendered resistant to 10 γ of isoniazid was not affected by the drug even at the higher concentrations employed.

DISCUSSION

A number of metabolites have been found to reverse the inhibitory action of isoniazid on the growth of tubercle bacilli. The term reversal, however, does not imply any particular mechanism whereby the action might take place. It simply means an antagonism of the growth inhibition otherwise caused by a certain concentration of isoniazid, irrespective of how this reversal is accomplished.

The nature of the isoniazid molecule makes difficult the interpretation of any data relating to its specific mode of action. Since this drug is a strong complexing agent because of its hydrazide grouping, one must be cautious in designating a specific site as the primary point of attack in the organism. It is quite probable that isoniazid inhibits multiple mechanisms in the cell simultaneously within a narrow range of concentrations. For this reason, in any study of this kind, rigor-

ously controlled threshold concentrations of the drug should be employed in order to minimize the inherent difficulties involved.

In spite of these obstacles, however, it is tempting to formulate an hypothesis in which one of the primary sites of action of the drug is in those essential enzyme systems containing pyridoxal phosphate as a vital component. These systems consist of a metal-pyridoxal phosphate-enzyme-substrate complex and play a prominent role in transamination, decarboxylation, and other reactions important in amino acid metabolism. This could explain the ability of pyridoxal, pyridoxamine, and certain of the amino acid substrates to antagonize isoniazid activity in the tubercle bacillus. The ability of pyridoxine to neutralize the drug's activity in other systems has also been demonstrated (1–5), and Schaefer has observed the effect of keto compounds (6).

The role of guanine, nucleic acid, and vitamin B_{12} is more obscure. Reversal by these substances cannot necessarily be construed as furnishing a product of some blocked reaction. They may not alleviate the block directly but may facilitate the ability of the organism to survive by some alternative metabolic pathway. Not only is a free amino acid pool necessary for enzyme synthesis, but the formation of new enzyme molecules occurs only if ribonucleic acid is available or can be synthesized from the required purine or pyrimidine. There are a number of postulated theories for the metabolic action of vitamin B_{12} in protein metabolism, and more recently its role as an activator of adaptive enzymes has been demonstrated (7).

Inexplicable at this time, however, is the finding that the degree of resistance of tubercle bacilli to isoniazid is considerably reduced when pyridoxal is included with the drug in *in vitro* studies. Also of interest is the observation that at certain concentrations of isoniazid the higher concentrations of pyridoxal are less effective than lower ones. These findings may account for some of the discrepancies in the literature and may have some bearing on the findings of Grunberg and Blencowe (8) that pyridoxine does not antagonize the prophylactic effect of isoniazid in mice infected with *M. tuberculosis* H37Rv.

Although isoniazid was found to inhibit the respiration of tubercle bacilli, very high concentrations were required to demonstrate a depressant action. This attack on the catabolic processes in the organism is thus believed to be of secondary importance, with the primary site of action involving processes concerned with the synthesis of metabolites vital to the multiplication and proper functioning of the organism.

SUMMARY

A number of metabolites have been tested for their ability to antagonize the *in vitro* inhibitory effect of isoniazid on *M. tuberculosis* H37Rv.

Pyridoxal was the most effective antagonist studied. Others which demonstrated a similar effect include pyridoxamine, sodium pyruvate, α-ketoglutarate, vitamin B_{12}, guanine, nucleic acid, L-alanine, D-alanine, L-arginine, L-glutamic acid, D-glutamic acid, L-leucine, L-lysine, L-proline, L-tyrosine, and DL-valine.

Tubercle bacilli resistant to 10 γ of isoniazid per ml. are markedly inhibited by

the drug when it is tested in the presence of subinhibitory concentrations of pyridoxal.

High concentrations of isoniazid inhibit the endogenous respiration of tubercle bacilli. This can be overcome by pyridoxal or alpha-ketoglutarate, but not by pyruvate. The oxidation of pyruvate, lactate, acetate, glucose, and glycerol is also inhibited by isoniazid.

SUMARIO

La Neutralización de la Actividad de la Isoniacida en el Mycobacterium Tuberculosis por Ciertos Metabolitos

Varios metabolitos han sido comprobados en cuanto a su capacidad para antagonizar el efecto inhibidor *in vitro* de la isoniacida sobre el *M. tuberculosis* H37Rv.

El piridoxal fué el antagonista más eficaz estudiado. Otros, que mostraron efecto semejante, comprenden la piridoxamina, el piruvato de sodio, el α-queto-glutarato, la vitamina B_{12}, la guanina, el ácido nucleico, la L-alanina, la D-alanina, la L-arginina, el L-ácido glutámico, la L-leucina, la L-lisina, la L-pirolina, la L-tirosina y la DL-valina.

Los bacilos tuberculosos resistentes a 10 γ de isoniacida por ml. son inhibidos intensamente por la droga cuando se ensaya ésta en presencia de concentraciones subinhibidoras de piridoxal.

Concentraciones elevadas de isoniacida inhiben la respiración endógena de los bacilos tuberculosos. Esto puede sobrepujarse con piridoxal o alfa-quetoglutarato, pero no con piruvato. La oxidación del piruvato, del lactato, del acetato, de la glucosa y del glicerol también es inhibida por la isoniacida.

RESUME

Neutralisation de l'activité de l'isoniazide sur le Mycobacterium tuberculosi per certains métabolites

Un certain nombre de métabolites ont été testés pour évaluer leur aptitude à s'opposer *in vitro* à l'action inhibitrice de l'isoniazide sur le *M. tuberculosis* H37Rv.

Le pyridoxal était le plus efficace des antagonistes étudiés. Les autres composés qui exerçaient un effet antagoniste similaire sont les suivants: pyridoxamine, pyruvate de sodium, alpha-cétoglutarate, vitamine B_{12}, guanine, acide nucléique, L-alanine, D-alanine, L-arginine, acide L-glutamique, L-leucine, L-lysine, L-pyroline, L-tyrosine et DL-valine.

Les bacilles tuberculeux résistants à l'isoniazide à la concentration de 10 γ par ml. sont inhibés considérablement par celle-ci lorsqu'ils sont testés en présence de concentrations sub-inhibitrices de pyridoxal.

Les concentrations élevées d'isoniazide inhibent la respiration endogène du bacille tuberculeux. Ce phénomène peut être annulé par le pyridoxal ou l'alpha-cétoglutarate, mais pas par le pyruvate. L'oxydation du pyruvate, lactate, acétate, glucose et glycérol est également inhibée par l'isoniazide.

Acknowledgment

The writer wishes to express her appreciation to Mrs. Bettie Lou McCollum for her excellent technical assistance.

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TUBERCULOSIS IN THE DOG, WITH SPECIAL REFERENCE TO EXPERIMENTAL BRONCHOGENIC TUBERCULOSIS[1]

JOHN FRANCIS[2]

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INTRODUCTION

It has been recognized for a long time that the dog is rather resistant to experimental tuberculosis (1, 2). A selection of published data, chiefly from continental sources (3), showed that the incidence varied from 0.04 to 1.0 per cent of clinical cases and from 0.83 to 5.6 per cent of autopsy material.

Review of the Literature

Despite the high resistance of the dog to experimental infection and the low incidence of the disease, lesions in naturally occurring tuberculosis are often widely disseminated. Hjärre (4) comments on the fact that there is usually little tendency to healing and that the establishment of a primary complex is followed by rapid generalization. He observed primary complexes as incidental findings in only 9 of 5,000 autopsies. Three of these were complete and four were incomplete aerogenous complexes, one was an incomplete alimentary complex, and the other an incomplete cutaneous complex. Lovell and White (3) found no healed tuberculous lesions. Nieberle (4a) observed the healing of a primary pulmonary focus by fibrosis and hyalinization, but early generalization was the rule.

Tuberculous lesions in the dog usually resemble neoplasms, especially the sarcomata (5). Often the tubercles are grayish-white in color and are circumscribed. The lung lesions usually consist of grayish-red bronchopneumonic areas; some break down with the formation of cavities, which may open into the pleural cavity or communicate with a bronchus. Histologically the classical tubercle is not seen, and giant cells are very rare (3, 6).

It appears that there is considerable variation in the susceptibility of the various breeds. Thus Hjärre (4), who examined 175 tuberculous dogs, observed an incidence of 9 to 10 per cent in the Irish setter, fox terrier, bulldog, and other short-nosed breeds, whereas it was only 1.6 per cent in dachshunds. Of 22 cases observed by Lovell and White (3), 36 per cent were wire-haired fox terriers, whereas only 11 per cent of dogs received for autopsy were of this breed.

Vaccination (7), reinfection (8), and age (9) apparently have little influence on the development of pulmonary lesions following intrabronchial infection with tubercle bacilli. Following an experimental study, Colwell and Mills (10, 11) concluded that tuberculous dogs reacted well to tuberculin, but this is contrary to general clinical experience (12, 13, 13a), although the subcutaneous test gives rather better results than the intradermal test.

It was pointed out by Steenken (13b) that the experimental infections used for the assay of antituberculous drugs bear little resemblance to the disease in man, and particularly to the chronic bronchogenic lesions of phthisis.

Because of the evidence reviewed above, it was thought that the lesions in dogs produced by intratracheal infection with tubercle bacilli would have a

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greater resemblance to phthisis in man than tuberculous lesions in other animals. This was found to be correct, and the course of the disease is described in the present paper and its response to chemotherapeutic treatment in a subsequent paper (14).

MATERIALS AND METHODS

Except where otherwise stated, the human strain of tubercle bacilli (Weybridge C) and the bovine strain (A.N.5) were employed (15).

All of the dogs used were from a laboratory stock of beagles. When dogs were being infected intratracheally, they were anesthetized by the intravenous injection of pentobarbital sodium (Nembutal®) and in later experiments by thiopental sodium (Pentothal®) and were then infected with a suspension of tubercle bacilli grown on Löwenstein's medium for sixteen days. The suspension was made on a ball mill in 5 per cent serum-saline and contained 10 mg. of bacilli per ml.

When a dog was being infected, it was placed on its back on a board sloping at 25 degrees from the horizontal and a No. 14 needle on a sterile syringe was inserted into the trachea. The dog was turned on its right side; the sterile syringe was removed from the needle and replaced by the syringe containing the suspension of tubercle bacilli. The dose of 2 mg. (0.2 ml.) per kg. of body weight was then injected fairly rapidly. The dog was left on the board for two minutes and then removed, laid on its right side, and allowed to recover. If both lungs were to be infected, the process was repeated twenty minutes later with the dog on its left side. It was then left lying on that side.

Roentgenograms were taken with a Newton Victor K.5 portable roentgenographic unit. At first dogs were anesthetized with thiopental sodium for roentgenographic examination, but later it was found that this was not essential. Dogs were fastened to a sloping board by tapes on their front legs, so that the abdominal organs would move caudally. Photographs were taken at a distance of 2 ft. and exposures varied around 0.25 of a second.

The methods of pathologic assessment of lesions and assessment of the number of tubercle bacilli present have been described (16, 17).

OBSERVATIONS

The Susceptibility of Dogs to Intravenous Infection

Fourteen-day-old cultures of human and bovine tubercle bacilli were prepared and the same suspensions, suitably diluted, were used to infect all animals. Doses of 0.1 mg. and 0.002 mg. per kg. of body weight were given to 8 dogs and to all other animals except mice, which were given doses of 5.0 and 0.1 mg. per kg.

The average survival times of the dogs are shown in table 1, and of the other animals in table 2. It will be seen that, with the dose of 0.1 mg. of the bovine strain per kg., the rabbits died much sooner than the guinea pigs; but with the smaller dose the position was reversed. The dose of 0.1 mg. per kg. killed mice in approximately the same time as it killed guinea pigs, but it will be noted that the mice were infected intravenously and the guinea pigs intraperitoneally. From the results in numerous other experiments, it is obvious that with the smaller dose the mice would have lived much longer than the guinea pigs or rabbits. The human strain behaved very similarly to the bovine strain in mice and guinea pigs.

All of the dogs shown in table 1, except *Nos. 60* and *61*, were infected with the same suspensions as those used to infect the animals shown in table 2, but the results were very different. Only one dog (*No. 9*) infected with 0.1 mg. of human

TABLE 1

RESULT OF INFECTING DOGS INTRAVENOUSLY WITH VARIOUS STRAINS OF TUBERCLE BACILLI

Dog Number	Survival Time*	Strain	Dose	Result of Injecting a Suspension of Bronchial Lymph Nodes into 2 Guinea Pigs	Notes†
	days		*mg/kg*		
61	24	Human tubercle bacilli type *ex*, naturally infected dog *No. 59*	5.000	Not tested	Killed when in poor condition. Lungs were covered with coalescing miliary lesions (*see* text for details).
60	59		0.100	Not tested	Killed when in poor condition. Lungs were covered with coalescing miliary lesions (*see* text for details).
1	360	Bovine A.N.5	0.100	Negative	Faint white lesions on pleura. Roentgenogram of lungs showed no calcified foci. The liver, lungs, and bronchial nodes all contained foci of epithelioid or macrophage cells; but acid-fast bacilli were seen only in the bronchial lymph nodes.
2	69	Bovine A.N.5	0.100	Not tested	Appeared well when killed. Miliary foci similar to dog *No. 9* but less severe.
9	69	Human Weybridge C	0.100	Not tested	Killed when in poor condition. There were numerous miliary foci in the lung (T.B.3). No macroscopic lesions in other organs. But there were epithelioid foci (T.B.3) in the liver, spleen, and bronchial lymph nodes.
10	360	Human Weybridge C	0.100	Negative	Same as dog *No. 1*.
3	362	Bovine A.N.5	0.002	Negative	Very faint white lesions on pleura. Small faint white foci below capsule of kidney.
4	362	Bovine A.N.5	0.002	Positive	Very faint white lesions on pleura. Small faint white foci below capsule of kidney.
11	362	Human Weybridge C	0.002	Negative	Very faint white lesions on pleura. Small faint white foci below capsule of kidney.
12	362	Human Weybridge C	0.002	Negative	Very faint white lesions on pleura.

* All dogs were killed.

† "T.B." followed by a numeral indicates the number of bacilli as defined previously (16).

tubercle bacilli per kg. became ill and was killed: it had extensive tuberculous lesions. Dog *No. 2*, infected with 0.1 mg. of bovine bacilli per kg., was killed at the same time and, although it was not as sick as dog *No. 9*, it had fairly extensive tuberculous lesions. Dogs *Nos. 1* and *10*, infected with the same dose of bovine

TABLE 2

AVERAGE SURVIVAL TIME IN DAYS OF ANIMALS GIVEN THE SAME SUSPENSIONS OF TUBERCLE BACILLI AS THOSE USED TO INFECT THE DOGS SHOWN IN TABLE 1

Type of Tubercle Bacilli	Rabbit and Guinea Pig Doses	Rabbits	Guinea Pigs	Mice*
	mg/kg			
Bovine	0.100	19 (5)†	66 (4)	19.2 (30)
	0.002	122 (6)	76 (21)	67.0 (30)
Human	0.100		40 (4)	23.0 (30)
	0.002		62 (20)	65.0 (30)

* Mice received a dose fifty times larger than did the other animals. Rabbits and mice were infected intravenously and guinea pigs intraperitoneally.

† The figures in parentheses show the number of animals in each group.

and human bacilli, respectively, remained well and were killed 360 days after infection. On autopsy there were small, faint white areas on the pleura which were probably healed lesions. Roentgenographic examination did not reveal any calcified foci, and the emulsified bronchial lymph nodes did not produce tuberculosis in guinea pigs.

The 4 dogs given 0.002 mg. of human or bovine tubercle bacilli per kg. remained well for 360 days. There were faint residual lesions, as shown in table 1, and the bronchial lymph nodes of dog *No. 4* produced tuberculosis in guinea pigs. At autopsy roentgenographic examination of the organs of the foregoing dogs revealed no calcified foci in the lungs or bronchial lymph nodes.

Lesions produced by intrapulmonary infection with tubercle bacilli: The ability of the dog to overcome tuberculous infection is well illustrated by events in dog *No. 16*. This dog was infected through the thoracic wall into the diaphragmatic lobe of each lung with 5 mg. of human tubercle bacilli, contained in 0.25 ml. of 4 per cent starch, and the course of the disease was observed by frequent roentgenographic examination. After two weeks a well-defined cavity developed in each lung. The cavities then gradually became less well defined and after thirty weeks could be seen no more. The dog was killed sixty-two weeks after infection, and a roentgenogram of the lung after expansion and fixation by the intratracheal infusion of formalin showed a calcified focus in each lung but none in the bronchial lymph nodes. There was a faint, depressed scar in the dorsolateral surface of the diaphragmatic lobe of each lung which was surrouned by thickened pleura. White fibrous tissue extended in from the pleura to the calcified foci.

Lesions produced by intratracheal infection with tubercle bacilli: Twenty-four dogs, which did not receive chemotherapeutic treatment, were infected intratracheally into one lung with doses of approximately 2 mg. per kg. Only 2 of these dogs (*Nos. 68* and *70*) became ill and were killed 35 and 70 days after infection. Four dogs were infected with doses of 2 mg. per kg. into each lung and one (*No. 50*) became ill 56 days after infection and was killed.

Six of the 28 dogs were killed 5 to 74 days after infection, and the remaining 19 dogs were killed 100 to 120 days after infection, when cavities and fibrocaseous

FIG. 1. Roentgenogram of dog *No. 26* obtained 9 days after intratracheal infection with 10 mg. of tubercle bacilli into the right lung. The lower border of the consolidated cardiac lobe can be clearly seen, and this type of consolidation is illustrated in figure 4. (×0.5)

lesions were usually present in the lungs. Seven of the 28 dogs had pleurisy, which usually consisted of an adhesion between the lung and parietal pleura. There was pleural fluid in only one dog (*No. 68*); there were macroscopic lesions in the kidneys of 3 and in the liver of one dog.

Lesions found five to nine days after infection (dogs Nos. 32, 34, and 38): All of the dogs had extensive areas of firm white bronchopneumonic consolidation five to nine days after infection. The appearance obtained in a roentgenogram of such a lesion is shown in figure 1 (dog *No. 26*) taken nine days after infection. This was a control dog in Experiment I, but the lesion regressed and at autopsy 115 days after infection (which included roentgenographic examination), a slight area of calcification and a little necrotic tissue were found in the lung. The bronchial lymph nodes were only slightly or moderately enlarged and firm, but contained no necrotic or calcified areas. The cut surface of the enlarged nodes was firm and white.

Histologic examination of the lungs showed that in the firm white tissue the alveolar walls were thickened and the alveoli were filled with macrophage cells, alveolar phagocytes or epithelioid cells, and neutrophils; a little albuminous exudate was noted. The perivascular and peribronchial lymph spaces were greatly distended and usually contained few cells (figure 2, dog *No. 34*). The bronchial lymph nodes showed numerous areas of epithelioid cell proliferation lying among areas of lymphoid tissue. Tubercle bacilli were fairly numerous in the lungs and lymph nodes.[3]

Lesions 16 to 24 days after infection (dogs Nos. 34, 39, and 75): During the period of 16 to 24 days after infection, areas of necrosis, caseation, and cavitation developed in the lungs (figures 3 and 4) and bronchial lymph nodes. In general the histologic appearance was similar to that in dogs five to nine days after infec-

[3] The method of assessing the number of tubercle bacilli has been given by Francis (16).

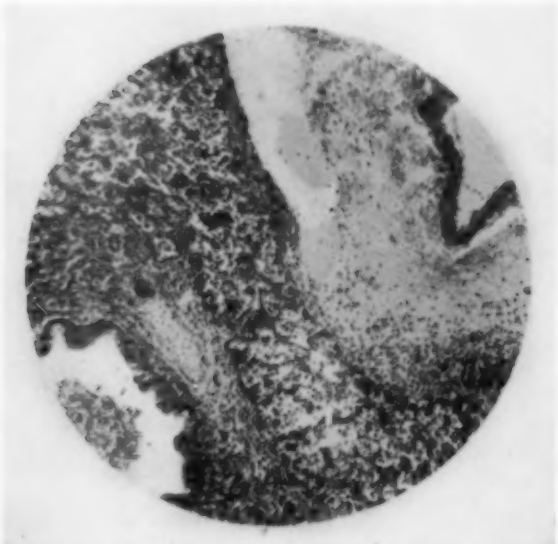

FIG. 2. Lung of dog *No. 34*, infected intratracheally with 10 mg. of human tubercle bacilli 16 days before autopsy. The perivascular lymph space is widely distended. The bronchus contains inflammatory cells. The alveolar walls are thickened and the spaces contain inflammatory cells. (H and E stain, ×85)

tion, but macrophage cells were being replaced by fibroblasts, and bacilli were definitely less numerous. There were early necrotic foci in the bronchial lymph nodes in which bacilli were more numerous. Areas of necrosis and caseation were infiltrated with polymorphonuclear cells.

Subsequent lesions to 120 days (22 dogs): Roentgenographic examination showed that resolution of the vascular white consolidated tissue usually commenced approximately 30 days after infection. This resolution often continued until the end of the experiment and caused some contraction which was particularly obvious in the apical lobe. Histologically these healed areas contained some fibrous tissue, foci of epithelioid and other inflammatory cells, which often contained globules of red acid-fast material, although sometimes this material was yellow. Bacilli were scanty except in adjoining necrotic foci. Persistent areas of caseation were usually surrounded by a considerable amount of fibrous tissue and occasionally contained calcified centers. The necrotic tissues often became liquefied, leading to the formation of cavities. All types of cavity walls were seen in dogs which were killed during the later stages of the disease. Some were lined by a layer of caseonecrotic tissue, containing acid-fast bacilli and surrounded by fibrous tissue. Others were lined by an almost complete layer of epithelium, and all stages between the two extremes were seen. The development of cavities was seen best in treated dogs and these cavities are described in a subsequent paper (14). The great thickening of the pleura which often occurred is evident in figure 3.

FIG. 3. Lung of dog *No. 75*, infected intratracheally with 10 mg. of human tubercle bacilli 24 days before autopsy. White consolidated apical and cardiac lobes and a few small lesions in the diaphragmatic lobe of the left lung may be noted. The right bronchial lymph nodes are enlarged. (×0.8)

FIG. 4. Section of lungs of dog *No. 75* (*see* figure 3). There are ragged cavities in the white consolidated cardiac lobe which is fused with the apical. There are a few lesions in the diaphragmatic lobe. (×1.1)

The lesions in dogs *Nos. 50, 68,* and *70* continued to advance and the dogs were killed when ill 56, 76, and 35 days after infection. They had extensive lesions of tuberculosis which in dog *No. 68* resembled those seen in natural tuberculosis (18).

Virulence of a Canine Strain

After a number of experiments had been performed (14), it was thought that the general mildness of the disease in dogs might be due to the use of an old laboratory strain of the tubercle bacillus (Weybridge C), although this strain was fully virulent in other animals. A culture of tubercle bacilli was isolated from the lung of a naturally infected dog (*No. 59*) which had bilateral bronchopneumonic lesions with cavities in both lungs (18). The bronchial lymph nodes were enlarged, soft, and necrotic, and there was extensive dry pleurisy with numerous adhesions and extensive pericarditis. Multiple lesions were noted in the abdominal cavity, liver, and kidneys.

The culture from dog *No. 59* was used to infect mice, guinea pigs, and rabbits 40 days later, after one subculture had been made. The results shown in table 3 indicate that it was a virulent human strain. Dog *No. 61* was infected intravenously with 5 mg. of bacilli per kg. and was killed 24 days later when in very poor condition (table 1). The lungs were covered with coalescing lesions rather smaller than those shown in figure 5. The bronchial lymph nodes were enlarged and caseous; they were infiltrated with epithelioid cells and fairly numerous giant cells. The liver was yellow and covered with large pale areas; it was infiltrated with epithelioid foci. The spleen was covered with small, translucent foci consisting of epithelioid cells and a few giant cells, but the kidneys appeared normal macroscopically and histologically.

Dog *No. 60* was infected intravenously with 0.1 mg. of bacilli per kg. It was killed after 57 days when in poor condition. The lungs were covered with miliary tubercles (figures 5 and 6) and other lesions were similar to those in dog *No. 61*.

The Weybridge C strain of the tubercle bacillus was reisolated from dog *No. 68*, in which the disease was particularly severe (*see* above). A young subculture of the strain isolated from this dog, 136 days previously, and a young subculture of the canine strain isolated from dog *No. 61*, 261 days previously, were used to infect 4 dogs approximately six months old. All dogs were infected by the intratracheal route in the right lung in the standard manner. One dog received 2 mg. per kg., and the other, 0.2 mg. per kg. of each strain. The results are shown in

TABLE 3

INFECTION OF VARIOUS ANIMALS WITH A STRAIN OF TUBERCLE BACILLI OBTAINED FROM DOG NO. 59*

Animal†	Number	Dose	Survival Time‡	Notes
		mg/kg	*days*	
Mice	20	5.00	D 19 (average)	Lesions in lungs
Guinea pigs	2	0.10	D 70 and D 79	Extensive lesions of tuberculosis
Guinea pigs	2	0.01	D 49 and D 98	Extensive lesions of tuberculosis
Rabbits	2	0.10	D 14 and K 123	Nonspecific; fairly numerous foci in lung
Rabbits	2	0.01	K 123	Few residual foci in lungs of both

* Rabbits and mice were infected intravenously and guinea pigs intraperitoneally.

† These results showed that the strain was highly virulent, and of human origin.

‡ D = dead. K = killed.

FIG. 5. Lung of dog *No. 60*, infected intravenously with 0.1 mg. per kg. of a strain of human tubercle bacilli obtained from dog *No. 59*. It died 56 days later. The lungs contained numerous coalescing lesions and small cavities. These lesions give the surface of the lung a mottled appearance; enlarged bronchial lymph nodes may also be noted. (×0.9)

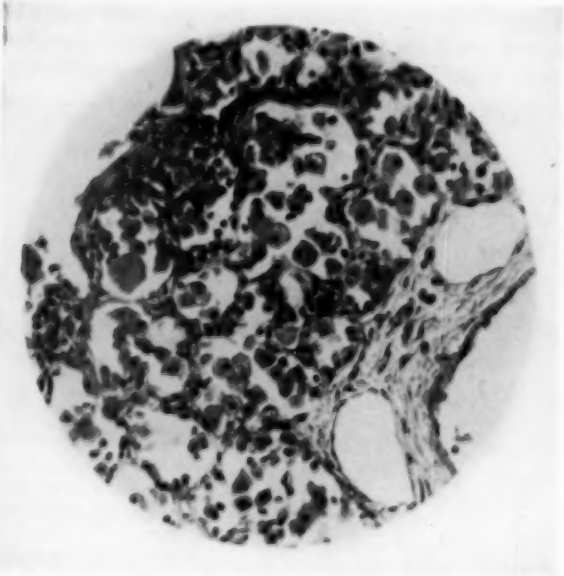

FIG. 6. Lung of dog *No. 60* (*see* figure 5). The wall of a blood vessel can be seen on the right. The alveoli contain large alveolar phagocytes and the alveolar walls are thickened. (H and E stain, ×250)

TABLE 4

THE RESULTS OF INOCULATING TWO STRAINS OF TUBERCLE BACILLI INTO THE RIGHT LUNG OF DOGS BY THE INTRATRACHEAL ROUTE*

Dog Number	Dose of Tubercle Bacilli	Lungs				Bronchial Lymph Nodes			
		Area Affected	Degree of Caseation	Extent of Cavitation	Total Score	Degree of Enlargement	Degree of Caseation	Extent of Cavitation	Total Score
				Strain *ex* Dog *No. 61*					
	mg. kg.								
113	0.2	2	2	0	4	1	1	0	2
114	2.0	5	2	5	12	2	1	3	6
				Strain *ex* Dog *No. 68*					
115	0.2	0	0	0	0	0	0	0	0
116	2.0	4	2	1	7	2	1	1	4

* Strain *ex* dog *No. 61* was a natural canine strain of human origin, reisolated from dog *No. 61*.

Strain *ex* dog *No. 68* was the Weybridge C strain reisolated from dog *No. 68*, in which it had produced very severe lesions.

All dogs were killed 130 days after infection.

table 4 and are similar to those obtained in other experiments (14). The disease was rather more severe in the dogs infected with the canine strain, but there was certainly not a tenfold difference.

The Effect of Nutrition on Susceptibility to Tuberculosis

Two litters of 6 dogs four months old were used; there were 3 dogs from each litter in each group. One group of dogs was given "ice cream" wafer biscuits soaked in water instead of the "puppy" meal which the animals had been receiving; the writer had been informed that the amount of protein in these biscuits was low, but subsequent analysis revealed 12 per cent protein. The other group continued with "puppy" meal and the dogs ate up to a maximum of 0.3 kg. a day. They were gradually changed over to "hound" meal and eventually ate up to 0.2 kg. a day in addition to about 0.3 kg. of meat. The dogs on the poor diet eventually ate about 0.4 kg. of wafer biscuits a day, but after eight days they began to have fits which were severe in some dogs. Due to deaths in dogs on the poor diet, the numbers in the group had to be reduced and by the thirty-eighth day the diets were standardized as follows:

Good diet: 0.2 kg. of hound meal, 0.3 kg. of raw meat, 2 gm. of liver, daily; 0.28 mg. of aneurin per kg. twice a week, 70 international units of vitamin D, and 140 international units of vitamin A per kg. once a week.

Poor diet: Wafer biscuits 0.3 kg., 2 gm. of liver, daily, and 0.18 mg. of aneurin per kg. of body weight twice a week.

These diets were continued until the one hundred and fifty-fifth day, when the 3 dogs on the good diet and 3 of the 4 dogs on the poor diet were infected into one lung with 2 mg. of human tubercle bacilli per kg. of body weight. One dog on the poor diet was left uninfected to serve as a "control" in case all of the other dogs on the poor diet succumbed rapidly following infection.

At the time of infection the average weight of the dogs on the poor diet was 10.2 kg. and of those on the good diet, 13.0 kg. There was no difference in the

TABLE 5

THE EFFECT OF DIET ON TUBERCULOSIS IN THE DOG

Dog Number	Time* to Death in Days	Macroscopic Pathologic Assessment†							Gain in Weight After Infection	Sedimentation Rates at Various Intervals After Infection			
		Pleurisy or Pleural Fluid	Lungs			Bronchial Lymph Nodes	Liver‡	Total Scores		Average of 3 Determinations, 7 to 21 Days	Average of 4 Determinations, 28 to 56 Days	Average of 4 Determinations, 70 to 126 Days	Average of All Determinations
			Area Affected	Caseation	Cavitation								
Treatment: Poor Diet													
									kg.				
130	145	0	2.0	0	0	0	0	2.0	1.36	22.0	8.00	4.25	10.45
132	147	1.0	2.0	0	3.0	1.0	0	7.0	−0.69	26.0	15.50	1.50	13.27
133	150	2.0	2.0	0	3.0	0	1.0	8.0	1.59	33.3	23.00	11.25	21.54
Average		1.0	2.0	0	2.0	0.3	0.3	5.7	0.75	27.1	15.50	5.70	15.16
Treatment: Good Diet													
134	145	2.0	3.0	2.0	2.0	0	0	9.0	0.02	36.3	34.00	14.00	27.36
135	147	0	3.0	2.0	0	0	0	5.0	2.11	13.7	7.25	2.25	7.19
136	150	0	2.0	1.0	0	0	0	3.0	0.68	20.0	19.75	6.00	14.82
Average		0.7	2.7	1.7	0.7	0	0	5.7	0.94	23.3	20.30	7.42	16.42

* All dogs were killed when in good health.

† For method of assessment, see section on technique.

‡ There were no lesions in the spleen or kidneys, and no peritonitis or ascites.

blood phosphate or protein concentrations in the two groups, but the average erythrocyte sedimentation rate of the dogs which received the good diet was 2.2 mm. and of the dogs on the poor diet, 5.3 mm. It may be seen from table 5 that the dogs on the good diet continued to gain weight more rapidly than those on the poor diet. The final average weights were 14 and 10.9 kg., respectively. There was, however, very little difference in the average blood sedimentation rates or in the final pathologic scores.

Tuberculin Tests

In the early experiments, dogs with varying degrees of tuberculosis were tuberculin tested with doses of PPD tuberculin, varying from 0.1 to 0.001 mg. The reactions were slight and irregular and indicated that the intradermal tuberculin test would be unreliable in the dog. Unfortunately no subcutaneous tuberculin tests were performed.

DISCUSSION

The present experiments show that the dog is considerably more resistant to the intravenous injection of tubercle bacilli than are the guinea pig and rabbit. An unpublished experiment showed that a dose of 0.1 mg. of the bovine strain, A.N. 5, of tubercle bacilli per kg. of body weight killed 4 calves in an average

time of 15.7 days, so that the dog is obviously much more resistant than the calf. On a milligram per kilogram of body weight basis, the dog probably has about the same susceptibility as the mouse to large doses of bacilli. It should be noted, however, that mice develop progressive pulmonary tuberculosis following the inhalation or intravenous administration of very small amounts of tubercle bacilli (19, 20, 21). It has been shown, however, that dogs may completely eliminate quite large doses given intravenously and may control infection following massive doses of bacilli given intratracheally or directly into the lung substance.

The results of tuberculin tests indicate that the dog develops only a low degree of allergy, and a given number of bacilli are less destructive to cells of the dog than of, say, the guinea pig. This type of resistance to the toxic action of tubercle bacilli is even more obvious in the mouse, in which hypersensitivity is low (15). The mouse has little ability to prevent the multiplication of tubercle bacilli, but the red acid-fast globules or yellow debris seen in the dog after intratracheal infection (and as seen in one case of natural tuberculosis (18)) indicate that the dog has great powers of destroying bacilli. The dog again differs from the mouse in that tuberculous lesions may be surrounded by a considerable amount of fibrous tissue. These three factors, i.e., low susceptibility to the toxic action of tubercle bacilli, considerable ability to destroy bacilli, and the ability to produce fibrous tissue, would appear to account for the dog's high resistance to tuberculosis. It appears that this resistance of the dog is a "native" resistance which is almost uninfluenced by previous infection or "vaccination" with attenuated or virulent tubercle bacilli (8). On the other hand, a large increase in resistance can be produced in the mouse (15).

It appeared that strains of tubercle bacilli from natural cases of tuberculosis in the dog might have specially high virulence for the dog, although these strains are known to be human or bovine strains of standard virulence in other respects (3). One strain isolated from a dog and shown to have the characteristics of a fully virulent human strain was, however, not obviously more virulent for dogs than the standard strain used in this laboratory when comparisons were made by intravenous or intratracheal injection.

The course of the disease was compared in dogs which received little but a diet of ice cream wafer biscuits and dogs which received hound meal and a liberal supplement of meat and vitamin D. There was no essential difference between the two groups of dogs, although those on the good diet eventually weighed 50 per cent more than those on the poor diet. The results therefore indicated that the variations in diet which occur under conditions of domestication are unlikely to explain the natural occurrence of severe tuberculosis in a species which is highly resistant to experimental infection. Again, differences in age do not appear to influence susceptibility to tuberculosis in the dog (9). In the present experiments, only 3 of 28 untreated beagles which were given approximately 10 mg. of tubercle bacilli intratracheally became obviously ill (14), whereas Mills, Barth, and Gunn (2) recorded a 30 per cent mortality in dogs obtained in a random manner and given 3 to 10 mg. of tubercle bacilli intrabronchially. It is known that there are considerable differences in the susceptibility of different breeds of dogs (3, 4) and it appears that, apart from the important influence of

the degree of exposure to infection, the occasional occurrence of progressive tuberculosis in dogs depends upon the existence of a few susceptible animals. The position is therefore not very different from that in man: most people overcome infection, but a small proportion develop progressive disease. As an example of a susceptible dog one may quote *No. 68* in the present experiments; the British Royal Commission (1) also found occasional dogs more susceptible than others. The important difference between man and the dog is that healed primary complexes are very seldom seen in the dog (4, 6). The results following intravenous, direct intrapulmonary, and sometimes intratracheal infection indicate that the absence of healed primary complexes in dogs is due to the fact that they may completely overcome tuberculous infection without there being any residual caseation or calcification at the site of the primary focus or in the regional lymph nodes. With the large doses of bacilli used in our experiments, slight lesions often remained, but it seems highly probable that in the natural course of events dogs might often overcome slight infection with tubercle bacilli with complete resolution of lesions.

Despite the differences between man and the dog, it was evident that the experimental disease in dogs had certain similarities to pulmonary tuberculosis in man, especially the post-primary type. The centers of large pneumonic areas underwent necrosis, caseation, and cavitation. These cavities were surrounded by fibrous tissue and their walls underwent varying degrees of healing. In most dogs the peripheral regions of the pneumonic areas underwent considerable resolution, but occasionally the disease progressed to a fatal termination.

SUMMARY

Different groups of dogs and other animals were infected intravenously with 0.1 and 0.002 mg., respectively, of virulent human or bovine tubercle bacilli per kg. The results indicated that the dog was equally susceptible to human and bovine strains, but was much more resistant to tuberculous infection than rabbits, guinea pigs, or calves. On a body weight basis, the dog probably had approximately the same susceptibility to intravenous infection with large doses of bacilli as mice, but it had much greater ability to overcome infection by small doses, which did not produce acute tuberculosis in either species.

A recently isolated canine strain of tubercle bacilli was no more virulent to dogs than the standard strain used in this laboratory. The course of the disease following the intratracheal injection of the laboratory strain was the same in dogs given a diet of biscuit alone or in dogs given a diet supplemented with vitamin D and a large amount of meat.

Tuberculin tests showed that the dog developed only a low and irregular degree of allergy, and pathologic examination indicated that tubercle bacilli had relatively low toxicity for canine tissues. These findings, combined with the considerable power of the dog's macrophages to destroy tubercle bacilli (as indicated by the presence of acid-fast globules or yellow debris) and its ability to form fibrous tissue around tuberculous lesions, probably account for the dog's high resistance to tuberculosis.

The course of tuberculosis following intratracheal infection with 2 mg. of tubercle bacilli per kg. is described. Human and bovine bacilli behaved in an essentially similar manner. Following infection, the alveoli became filled with macrophage and other inflammatory cells and the perivascular and peribronchial lymph spaces were distended. A cellular granulomatous pneumonia developed, followed by varying degrees of central necrosis, caseation, and cavitation. Calcification was rare, but there was often a vigorous fibrous tissue reaction. Usually, however, much of the consolidated area resolved, leaving somewhat contracted light yellowish-brown tissue. Histologically these areas contained some fibrous tissue, thickened alveolar walls, and foci of macrophage cells which frequently contained red or yellow acid-fast globules. The cavity walls often became smooth and were sometimes lined by epithelium.

A comparable series of changes occurred in the regional lymph nodes, but macroscopic hematogenous lesions were rare. Histologic lesions were common in the liver and occurred in the kidney.

SUMARIO

La Tuberculosis del Perro con Referencia Especial a la Tuberculosis Broncógena Experimental

A diversos grupos de perros y de otros animales se les infectó intravenosamente con 0.1 y 0.002 mg., respectivamente, de bacilos tuberculosos, humanos o bovinos, virulentos. Los resultados indicaron que el perro era susceptible por igual a las cepas humanas y bovinas, pero mucho más resistente a la infección tuberculosa que los conejos, cobayos o terneros. A base de peso vivo, el perro poseía con toda probabilidad aproximadamente la misma susceptibilidad que los ratones a la infección intravenosa con grandes dosis de bacilos, pero una capacidad mucho mayor para vencer la infección por dosis pequeñas, que no producían tuberculosis aguda ni en una ni en otra especie.

Una cepa canina de bacilos tuberculosos, recién aislada, no fué más virulenta para los perros que la cepa del laboratorio del A. La evolución de la enfermedad consecutivamente a la inyección intratraqueal de la cepa de laboratorio fué idéntica en los perros que recibieron un régimen dietético compuesto exclusivamente de galletas de las de perro y en los que recibieron una alimentación complementada con vitamina D y una gran cantidad de carne.

Las pruebas tuberculínicas demostraron que el perro no creaba más que una alergia de grado bajo e irregular y el examen histopatológico indicó que los bacilos tuberculosos poseían una toxicidad relativamente baja para los tejidos caninos. Estos hallazgos, combinados con la considerable facultad de los macrófagos del perro para destruir los bacilos tuberculosos (según indica la presencia de glóbulos o desechos amarillos ácidorresistentes) y la capacidad del animal para formar tejido fibroso alrededor de las lesiones tuberculosas, explican probablemente la alta resistencia del perro a la tuberculosis.

Describese la evolución de la tuberculosis consecutivamente a la inyección intratraqueal con 2 mg. de bacilos tuberculosos por kg. de peso. Los bacilos humanos y bovinos se portaron en forma semejante en el fondo. Después de la infección, los alvéolos se llenaron de macrófagos y otras células inflamatorias y se distendieron los espacios linfáticos perivasculares y peribronquiales. Se produjo una neumonía granulomatosa celular, seguida de esfacelo central, caseación y cavitación en varios grados. La calcificación fué rara, pero hubo a menudo una enérgica reacción de tejido fibroso. Sin embargo, por lo general se resolvió gran parte de la zona hepatizada, dejando tras sí tejido pardo-amarillento contraído. Histológicamente, estas zonas contenían tejido fibroso, paredes alveolares espesadas y focos de macrófagos que contenían frecuentemente glóbulos ácidorresistentes rojos o amarillos. Las paredes de las cavernas a menudo se alisaban y estaban a veces revestidas de epitelio.

Hubo una serie comparable de alteraciones en los ganglios linfáticos regionales, pero las lesiones hematógenas macroscópicas fueron raras. Las lesiones histológicas fueron comunes en el hígado y las hubo en el riñón.

RESUME

Tuberculose chez le chien, en particulier à l'égard de la tuberculose bronchogénique expérimentale

Différents groupes de chiens et autres animaux furent inoculés avec, respectivement, des doses de 0.1 et 0.002 mg de bacilles tuberculeux. Les résultats indiquent que la sensibilité du chien aux bacilles tuberculeux virulents humains et bovins est semblable, mais cet animal s'est montré beaucoup plus résistant à l'infection tuberculeuse que le lapin, le cobaye ou le veau. En prenant le poids pour base, le chien présentait probablement à peu près la même sensibilité à l'inoculation intraveineuse de fortes doses de bacilles que la souris, mais il possédait une aptitude beaucoup plus grande à résister à l'infection par les doses faibles, celles-ci ne produisaient la tuberculose aiguë dans aucune de ces deux espèces.

Une souche de bacille tuberculeux canin récemment isolée ne fut pas plus virulente pour les chiens que notre souche standard. Le cours de l'affection succédant à inoculation intratrachéale de notre souche standard était le même chez le chien recevant uniquement une ration de biscuit que chez le chien recevant en supplément de la vitamine D et une grande quantité de viande.

Les épreuves à la tuberculine montraient que le degré d'allergie présenté par le chien était faible et variable et les examens anatomo-pathologiques indiquaient que la toxicité du bacille tuberculeux pour les tissus du chien était relativement faible. Ces résultats, combinés au pouvoir considérable de destruction des bacilles tuberculeux que les macrophages du chien possèdent (comme l'indique la présence de cellules acido-résistantes ou de débris jaunes) et l'aptitude du macrophage a former du tissu fibreux autour des lésions tuberculeuses, constituent l'explication possible de la résistance du chien à la tuberculose.

Le cours de la tuberculose succédant à l'inoculation intratrachéale d'une dose de bacilles tuberculeux de 2 mg/kg est décrit. Les bacilles humains et bovins se sont comportés sensiblement de la même manière. A la suite de l'inoculation, les alvéoles se sont remplies de macrophages et d'autres cellules de type inflammatoire et les espaces périvasculaires et péribronchiques se sont distendus. Il se développe une pneumonie avec nodules tuberculeux avec ultérieurement divers degrés de nécrose centrale, caséification et formation de cavernes. La calcification a été rarement observée, mais il y avait souvent une importante réaction du tissu interstitiel. D'une manière habituelle, le processus inflammatoire se résolvait en grande partie laissant à sa place un tissu brun-jaunâtre clair légèrement rétracté. Histologiquement, on trouvait dans ces zones du tissu fibreux, des parois alvéolaires épaissies et des foyers de macrophages qui renfermaient fréquemment des cellules rouges ou jaunes acido-résistantes. Souvent, les parois des cavernes avaient acquis un aspect lisse et parfois s'épithélialisaient.

Des séries de modifications comparables se produisaient dans les ganglions lymphatiques régionaux, mais les lésions hématogènes macroscopiques étaient rares. Les lésions histologiques étaient fréquentes au niveau du foie et se produisaient parfois dans le rein.

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Notes

THE EFFECT OF THE "ANTI-ISONIAZID" SUBSTANCE PRODUCED BY MYCOBACTERIA ON THE CHEMOTHERAPEUTIC ACTIVITY OF ISONIAZID IN VIVO[1]

In 1951, Youmans and Youmans[2] reported that, when groups of mice were infected intravenously with different numbers of living virulent tubercle bacilli (strain H37Rv), a linear relationship was obtained when the logarithms of the infecting doses were plotted against the respective median survival times. This indicated that the tubercle bacilli in the mice were multiplying at a constant rate, regardless of the size of the infecting dose. Therefore, a change in the slope of the line produced by a chemotherapeutic agent would represent quantitatively the effect of the drug on the rate of multiplication of the tubercle bacilli *in vivo*.

With this possibility in mind, experiments were started in which mice were infected intravenously with varying amounts of tubercle bacilli and were treated with a small but highly effective oral dose of isoniazid. It was anticipated that the concentration of isoniazid employed would exert a proportionately smaller effect in mice infected with the larger inocula. This had been noted under similar experimental conditions with para-aminosalicylic acid.[3]

However, isoniazid in this concentration prolonged the survival times of the mice infected with the smaller inocula, but not the mice infected with the larger inocula. In order to prolong the survival time of the mice infected with the larger inocula, the amount of isoniazid in the diet had to be appreciably increased.

This finding suggested the possibility that the organisms might be excreting an "anti-isoniazid" substance into their environment which was capable of inactivating the drug. In a previous publication, the present writers reported that mycobacteria were able to form such a substance *in vitro*.[4] Filtrates of cultures of H37Rv, H37Ra, 607, and *Mycobacterium phlei* inactivated isoniazid 32 to 128 fold; and extracts of cultures of H37Ra and 607 decreased isoniazid inhibition 510 to 2,040 fold; results which indicated that 93.7 to 99.9 per cent of the isoniazid had been inactivated or destroyed *in vitro*.[4]

As a continuation of this work, experiments were planned in which filtrates and extracts of mycobacteria were injected into isoniazid-treated mice to determine whether the "anti-isoniazid" substance produced *in vitro* would inactivate isoniazid *in vivo*.

All mice were infected with the highly virulent strain H37Rv of *Mycobacterium tuberculosis* var. *hominis*. The organisms were grown as surface pellicles in flasks on the modified Proskauer and Beck medium, and the suspensions were made and standardized as described previously.[5] Both males and females of the "Strong A" strain of mice were used and ranged in weight between 18 and 22 grams. Groups of 20 mice were infected by the intravenous route with tubercle bacilli in amounts of 0.5, 1.0, 2.0, 3.0, and 4.0 mg., wet weight. Although the smaller inocula of H37Rv were not included in the experiments employing the larger concentrations of isoniazid, a control inoculum of 1.0 mg. was included in each experiment.

[1] This study was aided in part by a grant from Parke, Davis & Company, Detroit, Michigan.

[2] Youmans, G. P., and Youmans, A. S.: Am. Rev. Tubere., 1951, *64*, 534.

[3] Youmans, G. P., and Youmans, A. S.: Unpublished observations.

[4] Youmans, A. S., and Youmans, G. P.: Am. Rev. Tubere., 1955, *72*, 196.

[5] Youmans, G. P., and Karlson, A. G.: Am. Rev. Tubere., 1947, *55*, 529.

TABLE 1

THE SURVIVAL OF MICE INFECTED WITH VARIOUS INOCULA OF H37Rv WHEN TREATED WITH VARIOUS CONCENTRATIONS OF ISONIAZID

Concentration of Isoniazid in Diet	Median Survival Time in Days (ST_{50})				
	Size of Inoculum of H37Rv in Mg., Wet Weight				
	0.5	1.0	2.0	3.0	4.0
per cent					
0.002	20.5	14.5	11.5	10.8	4.75
0	16.0	14.5	11.0	8.8	2.8
0.004	37.0	38.0	8.6	3.0	1.8
0	17.0	13.5	8.1	2.0	1.5
0.008	66.0	60.0	49.0	3.3	2.9
0	16.0	13.5	8.6	2.8	2.0
0.016	89.0	78.0	59.0	47.0	2.2
0	17.0	14.0	10.2	6.0	2.4
0.032				67.0	80.0
0		15.5		7.2	3.5
0.064				63.0	79.0
0		13.0		4.9	2.4

The median survival times were estimated and the 95 per cent confidence limits calculated according to the method recommended by Litchfield.[6]

A mouse diet containing the desired concentration of isoniazid was prepared as described previously[7] and was administered to the mice for two weeks, starting the day before inoculation.

In the second part of the study, groups of 10 mice were given daily injections of either filtrates of cultures of H37Rv or cell-free extracts of the saprophytic strain, 607. These materials were prepared as described previously[4]; the filtrates were from fifty-day-old cultures of H37Rv and the extracts were made from seven- to twelve-day old cultures of strain 607. Injections of 0.5 ml. of the materials were made once a day intraperitoneally, or subcutaneously, beginning the day before the mice were placed on the diet, and were continued until the day after the diet had been removed. The mice in these experiments were infected with 1.0 mg. of H37Rv and were treated with a diet containing 0.004 per cent isoniazid.

In table 1 are listed the median survival times (St_{50}) of the mice treated with various concentrations of isoniazid and infected with varying amounts of tubercle bacilli. As the infecting dose was increased, twice as much isoniazid was needed to prolong significantly the median survival times of the treated mice.

In table 2 are given the results obtained with the mice which were infected with 1.0 mg. of tubercle bacilli and fed the diet containing 0.004 per cent isoniazid, when injected with materials containing the "anti-isoniazid" substance. The first experiment in which a filtrate of a culture of H37Rv was given was inconclusive, since statistically the difference between the median survival times of the two treated groups was barely significant

[6] LITCHFIELD, J. T., JR.: J. Pharmacol. & Exper. Therap., 1949, *97*, 399.

[7] YOUMANS, G. P.: Ann. New York Acad. Sc., 1949, *52*, 662.

TABLE 2

THE EFFECT OF "ANTI-ISONIAZID" SUBSTANCE ON THE ANTITUBERCULOUS ACTION OF ISONIAZID IN MICE

Experiment	Concentration of Isoniazid in the Diet	Material Injected	Median Survival Time (Ste) in Days (95 Per Cent Confidence Limits)		Recalculated Median Survival Time*		Per Cent Reduction in Median Survival Time†
	per cent						
A	0.004	H37Rv filtrate	31.0 (29.4–32.7)	S.‡	16.5	>	40.0
	0.004	0	42.0 (30.7–57.5)		27.5		
	0	H37Rv filtrate	13.5 (12.3–14.8)	N.S.§			
	0	0	14.5 (13.3–15.8)				
B	0.004	607 extract	36.0 (25.4–51.1)	S.	23.5	>	57.7
	0.004	0	68.0 (48.9–94.5)		55.5		
	0	607 extract	11.5 (10.5–12.7)	N.S.			
	0	0	12.5 (11.4–13.8)				
C	0.004	607 extract	13.0 (9.2–18.3)	S.	0	>	100.0
	0.004	0	57.0 (51.8–62.7)		44.0		
	0	0	13.0 (11.4–14.9)				
D	0.004	607 extract	39.0 (27.9–54.6)	S.	22.5	>	57.9
	0.004	0	70.0 (64.2–76.3)		53.5		
	0	607 extract	14.5 (13.1–16.1)	N.S.			
	0	0	16.5 (15.1–18.1)				
E	0.004	607 extract	33.5 (21.5–52.3)	S.	22.0	>	62.4
	0.004	0	70.0 (50.7–96.6)		58.5		
	0	607 extract	11.0 (9.6–12.7)	N.S.			
	0	0	11.5 (10.1–13.2)				
F	0.004	607 extract	19.0 (10.6–34.2)	S.	4.5	>	86.6
	0.004	0	48.0 (35.0–65.8)		33.5		
	0	607 extract	10.5 (9.9–11.1)	S.			
	0	0	14.5 (12.7–16.6)				

* Median survival times of the control infected mice subtracted from the median survival times of the treated mice.

† The difference between the recalculated median survival times of the treated mice divided by the median survival time of the control treated mice.

‡ S. represents a statistically significant difference.

§ N.S. represents a statistically nonsignificant difference.

The filtrate and extracts were injected intraperitoneally except in *Experiment B*, in which the material was given subcutaneously.

In the remaining five experiments, extracts of cultures of 607 were employed since these preparations inhibited the action of isoniazid to a much greater degree than did filtrates of cultures. Injections of the extracts into isoniazid-treated mice significantly shortened the median survival times when compared with the control isoniazid-treated mice. Furthermore, in only the one experiment, *F*, was there any indication that the extracts were toxic.

In order to compare experiments, the median survival time of the control infected mice was subtracted from the median survival times of the treated mice in each experiment. From these values the per cent reduction in median survival time by the "anti-isoniazid" present in the extracts could be determined. The extracts injected into the mice in experiments *B*, *D*, and *E* reduced the median survival times from 58 to 62 per cent. In experiments *C* and *F*, all or practically all of the isoniazid appeared inactivated since an 87 to 100 per cent reduction in the median survival time was obtained.

* * *

The data presented above show that an increase of 50 to 100 per cent in the numbers of organisms producing a tuberculous infection in mice will markedly reduce the therapeutic effect of isoniazid. How the tubercle bacilli are able to inhibit the suppressive activity of isoniazid cannot be definitely ascertained from the data at hand. Nevertheless, the demonstration that filtrates and extracts of mycobacteria which contain an "anti-isoniazid" substance will reduce *in vivo* the antituberculous activity of isoniazid suggests that this "anti-isoniazid" substance may be the responsible agent. An analogous situation does exist. Levaditi and associates[5] observed a decrease in the survival times of mice infected with staphylococci and treated with both penicillin and penicillinase as compared to the control mice treated with penicillin alone. It is well established also that infections produced by penicillinase producing micrococci may be resistant to therapy with penicillin.

In summary: The suppressive effect of isoniazid on an experimental tuberculous infection of mice was found to be inversely proportional to the size of the infecting dose of tubercle bacilli.

Culture filtrates and extracts of mycobacteria which contain an "anti-isoniazid" substance were found when administered parenterally to mice to appreciably reduce the suppressive effect of isoniazid on an experimental tuberculous infection of mice.

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[5] Levaditi, C., *et al.*: Compt. roy. soc. de biol., Paris, 1945, *139*, 381.

ISONIAZID SUSCEPTIBILITY, CATALASE ACTIVITY, AND GUINEA PIG VIRULENCE OF RECENTLY ISOLATED CULTURES OF TUBERCLE BACILLI[1]

In the relatively short period of two years there has accumulated a rather large and confusing literature pertaining to the pathogenic potentialities of isoniazid-resistant tubercle bacilli. The situation was clarified somewhat by Middlebrook's observation on the relationship between catalase activity and isoniazid resistance,[2] but there are still many questions left unanswered. A unique opportunity to answer some of these questions was offered by the collection in this laboratory of a large number of cultures from isoniazid-treated patients included in the United States Public Health Service Cooperative Investigation of Antimicrobial Therapy of Tuberculosis.[3] This report concerns itself mainly with the relationship of isoniazid susceptibility, catalase activity, and guinea pig virulence of 131 of these cultures. Data of similar nature but of limited extent have been reported by Neumayr, Morse, and Morse,[4] Peizer and Widelock,[5] and Viallier and Cayré.[6] In contrast to the situation in some of these studies, all of the patients in the present study received isoniazid in a daily dosage of 10 mg. per kg.

In this study, the sputum cultures were planted at the cooperating hospitals and were sent by mail to the Trudeau Laboratory when fully grown. Subcultures were then made in liquid Tween®-albumin medium and American Trudeau Society solid medium. Drug-susceptibility tests were performed by inoculating two double loopfuls from the liquid medium subculture onto slants of solid egg medium containing various concentrations of streptomycin or isoniazid. For the virulence tests, a representative sample of the two- to three-week subculture on solid medium was suspended in physiologic saline after lightly grinding in a mortar. After the clumps had settled, the suspension was adjusted to a standard turbidity corresponding to approximately 0.5 mg. (dry weight) of bacilli per ml., and each of 3 tuberculin-negative guinea pigs was inoculated subcutaneously in the inguinal region with 0.2 ml. Intracutaneous tuberculin tests were performed on each animal at least once, and all survivors were sacrificed three months after infection.

Catalase tests were carried out by adding a few milliliters of the reagent, consisting of equal parts of 30 per cent hydrogen peroxide and 10 per cent aqueous Tween® 80, to the fresh solid medium subculture. The test was considered to be positive when there was a prompt gross evolution of oxygen bubbles, and negative when there was either no bubbling or only a very slight reaction.

The degrees of virulence were defined as follows:

Virulent: At least one animal dead of tuberculosis within three months, or all animals showing advanced generalized disease when sacrificed.

Slightly attenuated: Gross disease of the internal organs but less extensive than in the above category, and no deaths from tuberculosis.

Highly attenuated: A local abscess at the site of inoculation with caseation of inguinal and iliac lymph nodes, but no more than an occasional tubercle in the lungs, liver, and spleen.

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[1] This study was aided by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, Public Health Service.

[2] Middlebrook, G.: Am. Rev. Tuberc., 1954, *69*, 471.

[3] Mount, F. W., and Ferebee, S. H.: Am. Rev. Tuberc., 1952, *66*, 632.

[4] Neumayr, R. B., *et al.*: Proc. Soc. Exper. Biol. & Med., 1955, *89*, 468.

[5] Peizer, L. R., and Widelock, D.: Am. Rev. Tuberc., 1955, *72*, 246.

[6] Viallier, J., and Cayré, R. M.: Compt. rend. Soc. de biol., 1955, *149*, 734.

Avirulent: Either no evidence of disease or, at the most, a local abscess at the site of inoculation with or without enlargement of the inguinal and iliac lymph nodes.

The classifications of isoniazid susceptibility were as follows:

Susceptible: No appreciable growth (less than 40 colonies) on the culture containing 0.1 γ of isoniazid per ml.

Slightly resistant: Growth equal to or only slightly less than the control on the culture containing 0.1 γ, but no appreciable growth on 1.0 γ per ml.

Moderately resistant: Growth equal to or only slightly less than the control on the culture containing 1.0 γ per ml., but no appreciable growth on 5.0 or 10.0 γ per ml.

Highly resistant: Growth equal to or only slightly less than the control on the culture containing 10.0 γ per ml.

Complete data are available for 131 cultures of tubercle bacilli obtained from a total of 74 patients, in 41 of whom only a single culture was available for evaluation for all three factors. In 18 patients, 2 cultures were evaluated; in 9, 3 cultures were tested; and 6 patients had 4 or more cultures tested before and during chemotherapy. All of the patients had advanced, bilateral, cavitary tuberculosis and had received initial chemotherapy with isoniazid-streptomycin, isoniazid-PAS, or isoniazid-streptomycin-PAS.

The dosages used were: 1 gm. of streptomycin twice weekly, 10 to 12 gm. of PAS by mouth daily; and 10 mg. of isoniazid per kg. by mouth daily. The 131 cultures studied represented part of a larger study to be reported later on the virulence of tubercle bacilli from patients on chemotherapy. The present cultures represent those processed during the latter half of 1954 plus such earlier ones as were still available and viable. The other cultures in the virulence study were not tested for catalase activity. The results are presented in table 1.

Catalase tests were also done on a much larger number of cultures not evaluated from the standpoint of guinea pig virulence, and the correlation between catalase activity and isoniazid susceptibility of 535 of these cultures (including the 131 cultures from table 1) is shown in table 2. The data presented in the two tables may be analyzed in a number of different ways.

It is clear that a negative catalase test was observed only in cultures resistant to isoniazid; 95 per cent of them showed a high degree of resistance and 5 per cent were moderately resistant. Catalase-positive cultures, on the other hand, were found equally

TABLE 1

CORRELATION OF ISONIAZID SUSCEPTIBILITY, CATALASE ACTIVITY, AND GUINEA PIG VIRULENCE OF 131 CULTURES

(Figures Indicate Number of Cultures)

Isoniazid Susceptibility	Catalase Activity	Guinea Pig Virulence			
		Virulent	Slightly Attenuated	Highly Attenuated	Avirulent
Susceptible (80)	Positive	76	4	0	0
	Negative	0	0	0	0
Slightly resistant (18)	Positive	11	4	0	3
	Negative	0	0	0	0
Moderately resistant (13)	Positive	3	2	1	3
	Negative	0	1	0	3
Highly resistant (20)	Positive	1	1	0	4
	Negative	0	2	1	11

TABLE 2

CORRELATION OF ISONIAZID SUSCEPTIBILITY AND CATALASE ACTIVITY OF 535 CULTURES, INCLUDING THE 131 FROM TABLE 1

Catalase Activity	Number of Cultures	Isoniazid Susceptibility							
		Susceptible		Slightly Resistant		Moderately Resistant		Highly Resistant	
		number	*per cent*	*number*	*per cent*	*number*	*per cent*	*number*	*per cent*
Positive	346	98	28	102	29	106	31	40	12
Negative	189	0		0		10	5	179	95
Total		98		102		116		219	
Per cent catalase-positive		100		100		91		18	
Per cent catalase-negative		0		0		9		82	

often in all of the categories of isoniazid susceptibility except in the highly resistant group which contained only 12 per cent. Looking at the correlation from the other side, 179 (82 per cent) of the 219 highly resistant cultures were catalase-negative and only 40 were catalase-positive, whereas all of the 200 drug-susceptible or slightly resistant cultures proved to be catalase-positive.

There was a clear-cut relationship between catalase activity and guinea pig virulence, as all but 3 of the 18 catalase-negative strains were avirulent or markedly attenuated; whereas, of the total of 113 catalase-positive strains, 102 (90 per cent) were virulent or only slightly attenuated. That there were exceptions, however, should be emphasized; 11 catalase-positive strains, only 4 of which were highly resistant to isoniazid, were avirulent or highly attenuated for guinea pigs. Moreover, 3 catalase-negative strains, all either highly or moderately isoniazid resistant, nevertheless maintained a fair degree of virulence for the guinea pig.

It may be seen from the data in table 1 that, in general, there was an inverse relationship between isoniazid resistance and guinea pig virulence, but there were outstanding exceptions. Three cultures of only slight resistance to isoniazid were nevertheless avirulent for guinea pigs, while there were 4 cultures which proved to be virulent or only slightly attenuated, despite a high degree of resistance to the drug.

Three patients (not included in the tables) consistently yielded cultures of "atypical" acid-fast bacilli which were chromogenic, avirulent for guinea pigs, and very strongly catalase-positive (more prompt and vigorous evolution of gas than is ordinarily noted with tubercle bacilli). The isoniazid susceptibilities of these strains varied from slightly to highly resistant. The organisms from 2 patients increased in isoniazid resistance during the course of therapy but still maintained a high degree of catalase activity. A similar retention of strong catalase activity was noted in 3 other "atypical" strains of human origin (2 photochromogens and 1 scotochromogen) and in a strain of the saprophyte, *Mycobacterium ranae*, made resistant to at least 100 γ of isoniazid per ml. by serial *in vitro* transfers in this laboratory.

* * *

Most of the cultures examined in the present study were probably not homogeneous with respect to the three characteristics studied, but instead consisted of mixtures in

[7] MIDDLEBROOK, G., *et al.*: Am. Rev. Tuberc., 1954, *70*, 852.

[8] OESTREICHER, R., *et al.*: Am. Rev. Tuberc., 1955, *71*, 390.

various proportions. Middlebrook and associates[7, 8] have maintained that the emergence of catalase-positive, isoniazid-resistant mutants in such mixtures from a patient's sputum may be prevented in large measure by initiating treatment with no less than 8 mg. of isoniazid per kg. daily. The following observations on initially treated patients who received 10 mg. of isoniazid per kg. daily with streptomycin are therefore worthy of note:

Patient F. L. yielded 3 cultures of tubercle bacilli from the twentieth to the sixty-fourth week of treatment which were moderately to highly isoniazid resistant, showed a doubtful or negative catalase test, and produced a limited amount of visceral disease in one or 2 of the 3 inoculated guinea pigs. The testing of individual colonies of one subculture revealed the presence of a mixture of catalase-positive and catalase-negative colonies, with the latter predominating. In addition, catalase-positive, isoniazid-susceptible, guinea pig-virulent organisms were recovered from the spleens of guinea pigs inoculated with 2 of the original cultures.

The cultures of tubercle bacilli from *Patient A. S.* remained catalase-positive during sixty-four weeks of therapy, although they exhibited first a moderate and then a high degree of isoniazid resistance (never more than slight streptomycin resistance). The fourteen-week and eight-month cultures had no guinea pig virulence, but each of the 3 cultures isolated from the eleventh month to completion of treatment produced a small amount of visceral disease in one or 2 of the animals. Organisms recovered from a guinea pig spleen three months after inoculation showed two of the characteristics of the original culture, namely, catalase activity and moderate isoniazid resistance, but exhibited no guinea pig virulence. If this original culture represented a mixed strain, it is difficult to explain why the catalase-positive guinea pig-virulent bacilli were not recovered from the animal's spleen. Seven months after isoniazid was stopped, a culture from this patient was still catalase positive and guinea pig avirulent, although only slightly resistant to isoniazid.

The tubercle bacilli obtained during the entire sixty-four weeks of treatment from *Patient E. K.* remained catalase positive and fully guinea pig virulent despite the appearance of a high degree of resistance to streptomycin and isoniazid. In this case the so-called "undesirable" mutants apparently constituted the vast majority of the population.

The relative frequency with which these catalase-positive isoniazid-resistant mutants appear in comparable groups of patients treated with the higher as opposed to the lower dosage of isoniazid remains to be studied. Another area for investigation is the behavior of artificial mixtures of catalase-positive and catalase-negative organisms. Preliminary experiments from this laboratory[9] indicated that, in order to produce gross visceral lesions in three months, guinea pigs must be infected subcutaneously with more than 1,000 (0.001 per cent) catalase-positive tubercle bacilli in the presence of a standard inoculum of approximately 100 million of the catalase-negative mutants.

* * *

One may question the adequacy of the described gross catalase test, particularly since two other groups of investigators[4, 5] used quantitative techniques. Neumayr and associates, measuring the liberated oxygen, observed variations in catalase activity from as high as 260 to as low as 23 microliters of oxygen per milligram among virulent cultures, whereas the 7 strains with catalase activity of 16 down to 0 microliters of oxygen were definitely attenuated for guinea pigs. Peizer and Widelock studied 44 strains of tubercle bacilli, all but 7 of which had significant resistance to isoniazid. Of the 24 strains exhibiting by their criteria either 50 or 100 per cent catalase activity, all were guinea pig virulent;

[9] Steenken, W., Jr., and Wolinsky, E.: Tr. Fourteenth Conference on Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 210.

12 of the 14 strains with only 10 or 25 per cent catalase activity were also virulent or only slightly attenuated; but all of the 6 completely catalase-inactive strains were avirulent. These estimations of virulence were made from Peizer and Widelock's table by the present writers' criteria. Nevertheless, their value is open to question since they are based on the findings in a single guinea pig inoculated with each strain. The great variability in amount of disease seen in individual animals of a group, each guinea pig having received an apparently identical inoculum, has been demonstrated repeatedly in this laboratory.

At any rate, the present studies indicate that it is only a markedly reduced endogenous catalase activity which is significant with respect to isoniazid resistance and guinea pig virulence and that the crude qualitative test should be sufficient for ordinary purposes.

In summary: From the data presented, it is reasonable to conclude that the finding of a negative catalase test in a culture of tubercle bacilli is significant in that it indicates reliably a moderate to high degree of isoniazid resistance and the probability of attenuation of virulence for guinea pigs. Three of 18 catalase-negative isoniazid-resistant strains of tubercle bacilli, however, showed only a slight attenuation of guinea pig virulence. This presumably resulted from the presence of a small but significant number of catalase-positive isoniazid-susceptible mutants. All degrees of isoniazid resistance or susceptibility may be noted among catalase-positive strains, and their virulence for guinea pigs cannot be accurately predicted. An uncontaminated culture of acid-fast bacilli which gives a very strong catalase reaction should be suspected of being either a saprophyte or an "atypical" acid-fast strain. The emergence of significant numbers of catalase-positive isoniazid-resistant mutants was noted in at least two patients who received initial therapy with 10 mg. of isoniazid per kg. daily.

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A BACTERIOLOGIC STUDY OF THE PLEURAL EXUDATE FOLLOWING SMALL RESECTIONS FOR PULMONARY TUBERCULOSIS

The technical features of cutting through lung tissue planes in small resections for pulmonary tuberculosis have been considered potentially hazardous because of the basic pathology of the disease. In view of the seriousness of postoperative complications involving tuberculous infection of the pleural space or chest wall, it was thought worth while to study the possible early pleural contamination by tubercle bacilli in operations of this type in order to evaluate present concepts of the disease as well as the postoperative complications encountered.

The present study involved the collection of all of the pleural exudate in sterile subaqueous drainage bottles following twenty-two consecutive pulmonary resections performed between August, 1954, and June, 1955, on 21 patients. Twenty-four-hour collections of the exudate were effected until the thoracic catheters were removed at an average of sixty hours. Sediments of centrifuged fluids were subjected to concentration with 4 per cent sodium hydroxide. Bacteriologic studies included microscopic examination of smear preparations stained by the Ziehl-Neelsen technique, culture on Löwenstein-Jensen and Petragnani media and, in some instances, guinea pig inoculation.

The operations described were performed on white, male patients between the ages of twenty-one and fifty-eight; the average age was thirty-five years. All of them had received antituberculous drug therapy averaging 9.6 months in duration; the range extremes were two weeks and four years. These men had positive skin reactions to purified protein derivative and 20 had demonstrated positive gastric or sputum cultures for *M. tuberculosis* prior to institution of antituberculous therapy. Cavitary lesions were shown roentgenographically in 19 of these patients and nodular disease was present in the remaining 2.

Surgically (table 1), the procedures performed were segmentectomies in 14 patients; subsegmental resection in 7, concomitant with decortication in 2, and extraperiosteal Ivalon® plombage in one; and left lobectomy in one patient. Removal of all palpable disease was accomplished in twenty of twenty-two resections; one patient included had staged bilateral resections.

The resected tissues in all instances showed fibrocaseous lesions which were consistent histologically with pulmonary tuberculosis. Cavitary lesions were verified grossly and microscopically in fifteen instances and in 4 patients caseous lesions were considered to represent filled cavities.

The results of bacteriologic studies of twenty-two resected tissues and corresponding pleural exudates may be seen in table 2. Among the resected tissues, twelve specimens were negative for tubercle bacilli on microscopy and culture; seven were positive on microscopy and negative on culture; one was negative on microscopy and positive on culture; and two were positive on both. *M. tuberculosis* and atypical acid-fast chromogens were recovered by culture in *Case 8*; however, acid-fast bacilli were not seen on microscopy. In a total of 4 guinea pig inoculations, all were negative for tubercle bacilli, as were corresponding smears and cultures.

Among the twenty-two pleural exudates studied, all were negative for acid-fast bacilli on microscopy. *M. tuberculosis* was recovered by culture in one instance (*Case 10*); positive smears and cultures were obtained from the corresponding resected tissue. Atypical acid-fast chromogens were recovered by culture in three instances. In a total of 9 guinea pig inoculations, all were negative for tubercle bacilli; with the exception of one smear, all corresponding smears were negative and all corresponding cultures were negative.

TABLE 1

SURGICAL DATA REGARDING RESECTIONS

Surgical Procedures	
Right superior segmentectomy	2
Right apical and posterior segmentectomy	9
Left upper lobectomy	1
Left apico-posterior segmentectomy	3
Subsegmental resections	10
Right	6
Left	4
Decortication	2
Extraperiosteal Ivalon® plombage	1
Residual palpable disease	2

TABLE 2

BACTERIOLOGIC STUDIES OF 22 RESECTED TISSUES AND CORRESPONDING PLEURAL EXUDATES

Cases	Bacteriology of Resected Specimen		Bacteriology of Pleural Exudate	
	Smear	Culture	Smear	Culture
22	9+	3+*	0	1+ 3+†

* *Case 8:* Atypical acid-fast chromogen and *M. tuberculosis*.
† Atypical acid-fast chromogen.

The particular case in which acid-fast bacilli were demonstrated was unusual, however, and clinical and operative technical features set it aside from the other cases so that a brief resumé is in order.

> *Case 10.* G. K., a thirty-year-old white male with marked rheumatoid arthritis and Marie-Strumpel spine, was readmitted for re-treatment after a long history of unsanctioned discharges from this and other institutions. The patient's diagnosis of pulmonary tuberculosis had originally been made in 1945. A left eight-rib thoracoplasty had been performed in 1946 and a right extrapleural paraffin plombage in 1947, both procedures having been accomplished at other hospitals. The patient had continued to discharge tubercle bacilli and these were considered to be coming from an 8 cm. cavity under the left thoracoplasty. In addition, the organisms recovered were the left thoracoplasty. In addition, the organisms recovered were found to be resistant to streptomycin, isoniazid, para-aminosalicylic acid, viomycin, and pyrazinamide. In spite of an extremely low respiratory reserve, as shown by clinical studies and pulmonary function tests, the patient was considered a possible salvage case and surgical extirpation was recommended.

During the operative removal of the left upper lobe, the large cavity was inadvertently invaded while an extrapleural dissection of the lobe was being performed. The specimen proved to be positive for *M. tuberculosis* on microscopy and culture. In addition, tubercle bacilli were cultured from the pleural exudate collected during the twenty-four hours following operation; however, bacilli were not demonstrated on microscopic examination of the fluid. The patient died of respiratory insufficiency approximately thirty hours after the surgical procedure.

In summary: The pleural exudates following twenty-two small resections for pulmonary tuberculosis were studied bacteriologically to determine possible contamination. Tubercle bacilli were cultured from the pleural fluids of one patient in whom distinct contaminations had occurred during the operation. Atypical acid-fast chromogens were isolated from three additional exudates. It is concluded that, except with direct invasion of active lesions, the danger of pleural contamination by tubercle bacilli is not potentiated by the opening of tissue planes which occurs in small resections for pulmonary tuberculosis.

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January 20, 1956

PURIFIED DIHYDROSTREPTOMYCIN

Soon after the introduction of streptomycin, clinical trials demonstrated that its prolonged use often produced disturbances of vestibular function and, occasionally, impaired hearing.[1, 2, 3] Dihydrostreptomycin was introduced in 1948[4, 5] because it was less toxic for the vestibular system, but this advantage proved to be offset by an increased tendency to cause hearing loss.[6] The ototoxicity of dihydrostreptomycin seemed to vary somewhat, depending upon the degree of chemical purity. Furthermore, other antimicrobials were discovered, such as hydroxystreptomycin and neomycin, which had a much more severe toxic action on the cochlea. For these reasons it seemed possible that the ototoxic effect of dihydrostreptomycin might be enhanced by the presence of other substances closely related to it but more toxic to the organ of Corti.

With this thought in mind, one of the major manufacturers[7] of dihydrostreptomycin prepared an especially purified batch of the drug by chromatographic and recrystallization techniques. The resulting product was in close conformance with the usual physical standards for crystalline dihydrostreptomycin sulfate but, more importantly, it was essentially devoid of any impurity, as indicated by paper-strip chromatograms. The potency as measured by microbial assay was 817 γ base per mg. of substance.

Field trials with this preparation were begun in the early summer of 1953 at three of the New York State tuberculosis hospitals. The protocol governing the study restricted the use of the preparation to previously untreated patients between the ages of fifteen and fifty years, inclusive, having, in so far as possible, normal audiograms. However, in order to obtain a sufficient number of cases, several patients showing spotty losses of 10 or 15 decibels at frequencies below 8,000 cycles per second and as much as 25 or 30 decibels at 10,000 cycles per second had to be accepted for the study.

Each patient was to receive the purified dihydrostreptomycin in a dosage of 1 gm. three times per week and para-aminosalicylic acid, 12 gm. daily, for a period of not less than four months. The total duration of therapy was to be governed by the clinical indications. Serial audiograms were taken at monthly (or in some cases bimonthly) intervals during the period of study.

Observations were made on 32 patients treated for five and one-half to twenty-one months. Twenty-four of them showed no significant audiometric change during or after treatment, while 8 developed some degree of hearing loss. A breakdown of the cases according to duration of therapy is shown in table 1.

Four other patients were excluded from the study because they received treatment for less than four months. Treatment had been discontinued in 3 cases when the patients left the hospital against medical advice, and in one case because of a drug reaction which occurred after twenty-one days of therapy. This patient was later successfully treated with streptomycin.

Details of the 8 cases in which hearing loss occurred are presented in table 2. The abbreviated audiograms represent the last measurements made, in some cases after the

[1] Chase, J. S.: Am. Rev. Tuberc., 1947, *56*, 418.
[2] Hinshaw, H. C., *et al.*: J.A.M.A., 1946, *132*, 778.
[3] Farrington, R. F., *et al.*: J.A.M.A., 1947, *134*, 679.
[4] Edison, A. O., *et al.*: Am. Rev. Tuberc., 1948, *58*, 487.
[5] Hobson, L. B., *et al.*: Am. Rev. Tuberc., 1948, *58*, 501.
[6] Mahady, S. C. F., *et al.*: Am. Rev. Tuberc., 1953, *68*, 238.
[7] Merck & Co., Inc., Rahway, New Jersey.

TABLE 1

INCIDENCE OF HEARING LOSS

Duration of Therapy	Number of Patients Retaining Normal Hearing	Number of Patients Developing Hearing Loss
months		
4–7	3	1
8–11	7	2
12–15	9	2
16–19	5	1
20–	—	2
Total	24	8

TABLE 2

SEVERITY OF HEARING LOSS

Patient (Sex, Age)	Onset of Hearing Loss	Duration of Dihydrostreptomycin Therapy	Additional Therapy, as Streptomycin	Abbreviated Audiograms					Hearing Loss for Speech (Fletcher)
				Months post Dihydrostreptomycin Therapy	Cycles per second				
					2,000	4,000	8,000	10,000	
	months	*months*							*decibels*
B.B. (F., 18)	15	18½	yes	2	−5	−10	25	35	0.0
H.C. (F, 43)	7	12	—	0	5	0	0	30	5.0
T.C. (M, 30)	8	8	yes	6	7	20	20	40	5.0
R.H. (F, 32)	7	21	yes	2	−5	−10	50	55	0.0
E.L. (M, 30)	5	7½	yes	13	15	15	65	65	10.0
G.P. (F, 29)	9	20	—	5	10	15	60	70*	12.5
C.S. (M, 20)	7	9½	—	1	0	30	45	50	5.0
S.S. (F, 27)	6	12½	yes	9	5	45	55	70*	7.5

* 70 per cent or more

patient had been switched to streptomycin for several months. They show the hearing loss for either ear, whichever is greater. In most cases the losses for the two ears were approximately equal. In addition, the "hearing loss for speech" has been estimated according to Fletcher's criterion (average of two smaller values of loss at 500, 1,000, and 2,000 cycles per second). From these figures it is clear that the losses were largely confined to the higher frequencies and that the frequencies essential for the perception of speech were relatively little affected.

For 4 patients (T. C., E. L., C. S., and S. S.) the losses shown in the table are slightly greater than at the time when dihydrostreptomycin was discontinued. Slight further hearing loss occurred after streptomycin had been substituted for dihydrostreptomycin. Audiograms of the other patients remained essentially stable after dihydrostreptomycin therapy was stopped.

While the numbers in this series are small, it is interesting to observe that the incidence of ototoxic complications (approximately 25 per cent) is the same as that reported by these same investigators in their original series published in August, 1953.[6] It should be emphasized that the earlier group of patients received daily dihydrostreptomycin therapy,

whereas the present group received dihydrostreptomycin three times per week. However, these findings tend to corroborate the original impression that the longer drug therapy is continued, the more likely it is that ototoxic manifestations will develop. Further, although dihydrostreptomycin may be discontinued at the time when auditory damage is first demonstrated, there may be further progression of the hearing loss in the subsequent months.

The number of patients in this series is small and the degree of hearing loss was relatively slight. The clinical effectiveness of the specially purified preparation seemed to be the same as that of standard forms of dihydrostreptomycin. Nevertheless, it is obvious that the special manufacturing techniques did not eliminate the ototoxic property of the drug.

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LIBERATION OF PEROXIDE IN THE BREAKDOWN OF ISONIAZID

It has been shown in several laboratories that isoniazid is slowly decomposed in a neutral buffer solution at 37°C. in the presence of oxygen. This breakdown is greatly influenced by pH—being very slow at pH 6, and greatly increased at pH 8—and is catalyzed by hemin and some metallic ions, especially manganese^{++}.[1, 2] A number of other factors influence the rate of decomposition. The main breakdown products have been shown to be *iso*nicotinic acid and di*iso*nicotinyl hydrazine.[3, 4] It does not appear to have been observed, however, that, when isoniazid breaks down in aqueous solution in the presence of oxygen, peroxide (presumably hydrogen peroxide) is formed in amounts approximately equimolar to the amounts of isoniazid which disappear.

When 100 γ of isoniazid per ml. were incubated in phosphate buffer (pH 7), containing 0.1 per cent citrate and 1 γ of manganese per ml. (as manganous chloride) at 37°C., 59 γ of isoniazid per ml. were destroyed in eighteen hours. (Isoniazid was measured by reaction of its hydrazine with dimethyl-aminobenzaldehyde.) The resulting solution contained an oxidizing agent which liberated iodine from potassium iodide, formed pertitanic acid from titanic sulfate, gave a positive reaction for peroxide with benzidine and peroxidase, and was catalytically destroyed by such peroxide-destroying agents as serum, cobaltous ions, and hemin. This peroxide was determined quantitatively by adding 1 ml. of an acid solution of titanic sulfate (prepared by heating excess titanium dioxide with concentrated sulfuric acid, diluting with an equal volume of water, and centrifuging to clear) to 3 ml. of the solution, and reading the resulting color at 400 mμ. Isoniazid did not interfere with the determination.

In the above experiment, approximately 0.3 micromoles of peroxide per ml. were formed for the destruction of 0.4 micromoles of isoniazid per ml. When hemin was used to catalyze the decomposition of isoniazid, no peroxide appeared.

* * *

This finding explains a number of observations recorded in the literature. The observation of Middlebrook and co-workers,[5, 6] that isoniazid-resistant strains of *M. tuberculosis* required for growth increased concentrations of peroxide destroyers in the presence of isoniazid, is explained by the fact that peroxide is formed in the medium on incubation of the isoniazid. It also provides an explanation for the finding that an originally catalase-negative isoniazid-resistant strain of H37Rv which had been trained to grow in the absence of serum, i.e., to recover some catalase activity, failed to grow when isoniazid was added.[7] The peroxide formed from the decomposition of the isoniazid, added to the peroxide normally present in the Proskauer and Beck medium,[8] was more than the organisms could deal with.

This formation of peroxide encourages one to suggest a hypothesis as to the mode of action of isoniazid. *M. tuberculosis* is susceptible to hydrogen peroxide in laboratory media (minimal inhibitory concentration approximately 20 γ per ml.), but inhibitory concentrations of isoniazid (approximately 0.05 γ per ml.) could not produce this concentra-

[1] Bönicke, R.: Naturwiss., 1954, *41*, 377.
[2] Lewin, E., and Hirsch, J. G.: Am. Rev. Tubere., 1955, *71*, 732.
[3] Albert, A., and Rees, C. W.: Biochem. J., 1955, *61*, 128.
[4] Krüger-Thiemer, E.: Naturwiss., 1955, *42*, 47.
[5] Cohn, M. L., *et al.*: Am. Rev. Tubere., 1954, *70*, 465.
[6] Cohn, M. L., *et al.*: Am. Rev. Tubere., 1954, *70*, 641.
[7] Barry, V. C., *et al.*: Am. Rev. Tubere., 1955, *71*, 785.
[8] Barry, V. C., *et al.*: Observations to be published.

tion of peroxide in the medium. It is suggested, however, that peroxide formed by a similar breakdown of isoniazid *within* the mycobacterial cell is responsible for the action of isoniazid.

It has been shown[9] that isoniazid (or its subsequent breakdown products) is accumulated in isoniazid-susceptible cells of *M. tuberculosis*. If this isoniazid is broken down within the cell in the same manner as outside the cell, the concentration of peroxide so formed could be greater than would be expected from the external concentration of isoniazid, and, in spite of the catalase normally present, even adequate to inhibit growth. Since the chemical breakdown of isoniazid is very sensitive to factors such as pH and the concentration of certain metal ions, and is dependent on free oxygen, it may be that the breakdown occurs almost exclusively in a special area in the cell, with the consequent production of a high local concentration of peroxide. If this locus were susceptible to peroxide, the small amount of catalase in the cell of *M. tuberculosis* would be no protection unless it were present at this site.

On this hypothesis it would be expected either that isoniazid does not penetrate resistant cells or that the conditions for its breakdown do not exist in these cells. This is in line with the results of Barclay and co-workers[9] which show that isoniazid is not accumulated in resistant cells.

It may be objected that this hypothesis does not take into account the important fact that most strains of *M. tuberculosis* resistant to more than 10 γ of isoniazid per ml. are catalase negative, and hence are more susceptible to external peroxide.[5, 6, 8] However, since loss of catalase does not *invariably* accompany resistance, especially lower degrees of resistance,[5, 6] and since it was possible to train a catalase-negative resistant strain to grow in a medium which contained peroxide without loss of isoniazid resistance,[7] it seems likely that there is no direct cause and effect relationship between loss of catalase activity and development of resistance. Instead, loss of catalase, when it occurs, seems to be an independent consequence of growth of the resistant strain for a number of generations in the presence of a comparatively high level of isoniazid. Reasons for this loss are being investigated.

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February 3, 1956.

[9] Barclay, W. R., *et al.*: Am. Rev. Tuberc., 1954, *70*, 784.

[10] The writer would like to thank the Director of the Laboratories, Dr. V. C. Barry, for stimulating discussion and for help in the drafting of this Note; and to thank his colleagues, Miss Joan Denneny and Dr. M. L. Conalty, for collaboration in much of this work.

ENHANCING EFFECT OF ADJUVANTS ON THE ANTITUBERCULOUS IMMUNITY ELICITED IN MICE BY METHANOL EXTRACTS OF TUBERCLE BACILLI

It has been shown elsewhere that the resistance of mice to experimental tuberculosis can be consistently increased by intraperitoneal injection of phenol-killed, acetone-washed tubercle bacilli[1, 2]. Whatever their degrees of virulence, all human and bovine strains of tubercle bacilli so far tested have proved capable of eliciting immunity when used under these conditions. There is evidence, however, that the killed cells differ in immunizing effectiveness, as well as in toxicity, depending upon the strain of bacilli and the age of the culture used for the preparation of the vaccine. On the basis of preliminary comparative studies, it has been found that reproducible results can be obtained by preparing the vaccine from young cultures (two to three weeks) of a certain substrain of BCG (BCG-P obtained through the courtesy of Dr. J. Aronson, Henry Phipps Institute, Philadelphia).

The immunizing activity of the killed cells of BCG resides in a fraction which is soluble —or at least dispersible—in hot methanol[3, 4]. The active material, separated by prolonged extraction with methanol at 55° C., can be obtained in a stable desiccated form by evaporation of the solvent. It is not soluble in aqueous media, but can be dispersed as a stable colloidal solution in water. As was to be expected, the crude methanol extract has now been found to consist of a mixture of several different materials, some of which are devoid of protective activity. No knowledge is as yet available of the chemical nature of the active material. Nor is it known whether it is related to the immunizing fraction obtained in the form of particulate material (mitochondria-like) by differential centrifugation from tubercle bacilli ground up with powdered glass[4].

In a preceding communication, some evidence was presented suggesting that the immunizing effectiveness of the methanol extract, and the duration of the immunity that it elicits, could be markedly increased by injecting the material in association with certain adjuvants[3]. Much additional information on this point has recently been obtained; a few of the new findings will be summarized here.

Mice, 4 weeks old, were vaccinated by the intraperitoneal route with a mixture of methanol extract and of adjuvant prepared as follows: 125 mg. of the extract were resuspended with a Teflon grinder in 5 ml. of water and emulsified with 0.75 ml. of arlacel A and 4.25 ml. of bayol F oil. The emulsion was injected in a dose of 0.1 ml. corresponding approximately to 1.25 mg. of extract. The mice of the control group received 0.1 ml. of the adjuvant mixture (5 ml. of water, 0.75 ml. of arlacel A and 4.25 ml. of bayol F oil) without methanol extract.

Each group of mice was divided into four subgroups of 12 animals which were infected either 46 days or 91 days after vaccination. Half of the animals received by the intravenous route 0.2 ml. of a ten-day-old culture of the virulent bovine strain MV in Tween®-albumin medium. The other half of the animals received 0.2×10^{-3} ml. of the same culture.

The cumulative numbers of deaths at different intervals of time in the four groups are presented in table 1.

[1] Dubos, R. J., *et al.*: J. Exper. Med., 1953, *97*, 221.
[2] Weiss, D. W., and Dubos, R. J.: J. Exper. Med., 1955, *101*, 313.
[3] Weiss, D. W., and Dubos, R. J.: J. Exper. Med., 1956, *103*, 73.
[4] Youmans, G. P., Millman, I., and Youmans, A. S.: J. Bacteriol., 1955, *70*, 557.

TABLE 1

PROTECTIVE EFFECT OF METHANOL EXTRACT INJECTED WITH ARLACEL OIL ADJUVANT

Methanol Extract	Time Between Vaccination and Infection	Infective Dose*	Cumulative Numbers of Deaths at Indicated Times After Infection*					
			3 Weeks	4 Weeks	5 Weeks	6 Weeks	7 Weeks	8 Weeks
mg.	*days*	*ml.*						
1.25	46	0.2	2	9	12			
0	46	0.2	6	11	12			
1.25	91	0.2	0	2	4	6	9	10
0	91	0.2	2	6	7	10	11	12
			5 Months	6 Months	7 Months	8 Months	9 Months	10 Months
1.25	46	0.2×10^{-3}	2	2	2	6	7	8
0	46	0.2×10^{-3}	6	9	9	10	11	12
1.25	91	0.2×10^{-3}	0	0	1	1	1	1
0	91	0.2×10^{-3}	2	2	4	5		6

* Of 12 mice infected intravenously with bovine culture MV.

It is apparent in table 1 that administration of the methanol extract-adjuvant mixture resulted in a prolongation of life in the four groups of infected animals, whether the infective dose was large or small and whether challenge infection was carried out 46 days or 91 days after vaccination.

In another experiment of the same pattern, the interval between vaccination and infection was extended to 168 days, and the resistance elicited by the methanol extract-adjuvant mixture was compared with that resulting from BCG vaccination.

For this purpose a number of mice received by the intravenous route 0.2×10^{-3} ml. of a six-day-old culture of BCG-P in Tween®-albumin medium. It was known from earlier studies that this technique of vaccination elicits in mice a high level of antituberculous immunity lasting many months. Other groups of mice received intraperitoneally either methanol extract (1.25 mg.) with arlacel-bayol oil mixture or the adjuvant alone.

All mice in this experiment were infected intravenously with 0.2 ml. of the bovine culture MV. The infective dose was administered either 42 days, 84 days, or 168 days after vaccination.

The cumulative numbers of deaths in each group at different periods of time after infection are presented in table 2.

The results presented in table 2 confirm and extend the conclusions derived from the preceding experiment. It may be seen that the mice vaccinated with methanol extract in association with arlacel-bayol oil adjuvant still exhibited a marked increased resistance to virulent tuberculous infection when tested 168 days after vaccination. Furthermore, in this experiment as well as in others not reported here, the immunity elicited by the methanol extract-adjuvant mixture was of the same order as that elicited by vaccination with living BCG.

It has been mentioned in an earlier publication that certain lipids of biologic origin have the ability to enhance the protective effect of the methanol extract.[1] Particularly effective in this respect are the endotoxins of gram-negative bacilli—the lipopolysaccharide somatic antigen of typhoid bacilli, for example. More recent experiments have revealed that pertussis vaccine injected in association with the methanol extract can also

TABLE 2

PROTECTIVE EFFECT OF LIVING BCG VS. METHANOL EXTRACT INJECTED WITH ARLACEL OIL ADJUVANT

Vaccination (Intraperitoneally)	Time Between Vaccination and Infection	Cumulative Numbers of Deaths at Indicated Times After Infection*							
		3 Weeks	4 Weeks	5 Weeks	6 Weeks	7 Weeks	8 Weeks	9 Weeks	10 Weeks
	days								
BCG (living)	42	2	7	9	10	12			
M.E.† and adjuvant	42	0	10	11	12				
Adjuvant alone	42	5	10	12					
BCG (living)	84	1	2	5	7	9	10	10	11
M.E.† and adjuvant	84	2	3	3	7	12			
Adjuvant alone	84	7	8	10	11	12			
BCG (living)	168	2	2	3	4	7	8	10	11
M.E.† and adjuvant	168	1	1	1	2	5	8	10	11
Adjuvant alone	168	4	7	7	8	9	9	12	

* Of 12 mice infected intravenously with 0.2 ml. of bovine culture MV.
† 1.25 mg. methanol extract.

TABLE 3

PROTECTIVE EFFECT OF METHANOL EXTRACT INJECTED WITH PERTUSSIS VACCINE

Vaccination (Intraperitoneally)		Time Between Vaccination and Infection	Cumulative Numbers of Deaths at Indicated Times After Infection*					
Methanol Extract	Pertussis Vaccine		5 Weeks	6 Weeks	7 Weeks	8 Weeks	9 Weeks	10 Weeks
mg.	*ml.*	*weeks*						
0.5	0.05	10	0	2	2	4	6	8
0.5	0	10	6	6	8	9	10	—
0	0	10	8	9	9	10	—	—

* Of 10 mice infected intravenously with 0.2 ml. of bovine culture MV.

enhance markedly the resistance of mice to experimental tuberculosis. This finding is illustrated in the following experiment.

A group of 12 mice received intraperitoneally 0.5 mg. of methanol extract in suspension in water. A second group received the extract in association with 0.05 ml. of pertussis vaccine. This vaccine was a commercial preparation distributed for human use by Lederle Laboratories, Pearl River, New Jersey. All animals were infected intravenously with 0.2 ml. of bovine culture MV in Tween®-albumin medium ten weeks after vaccination. The cumulative numbers of deaths in the three groups at various intervals of time after infection are presented in table 3. It may be mentioned in passing that similar results have been obtained with another preparation of pertussis vaccine (not packaged for human use), obtained through the courtesy of Dr. J. Munoz of Sharp & Dohme, Glenolden, Pennsylvania.

It is apparent from the results presented in table 3 that mice having received both methanol extract and pertussis vaccine exhibited a marked increase in resistance when challenged ten weeks after vaccination. It must be pointed out, however, that the interpretation of these findings is complicated by another fact discovered recently.[5] In brief, it has been found that injection of pertussis vaccine alone does increase markedly the resistance of mice to experimental tuberculosis; the duration of this protective effect is dependent upon the amount of vaccine injected, and also upon host factors not yet completely understood. Further experiments with multiple controls will be needed before it becomes possible to determine more precisely the mechanism through which pertussis vaccine enhances the protective effect of the methanol extract.

In summary: The cells from young cultures of BCG, killed with 2 per cent phenol and washed with acetone, have been used as starting material for studies on vaccination of mice against experimental tuberculosis. An active fraction free of bacillary bodies has been separated in a soluble (or dispersed) state from the killed bacterial cells by prolonged extraction with hot methanol. The crude fraction recovered after evaporation of the methanol is insoluble in aqueous media, but readily dispersible as a stable colloidal suspension. Mice receiving by the intraperitoneal route small amounts of this fraction (of the order of 1 mg.) consistently develop within two weeks an increased resistance to infection with bovine and human tubercle bacilli.

The immunizing effect of the methanol extract can be enhanced and prolonged by the use of certain unrelated adjuvants. The following substances have been found active in this respect: (*a*) a mixture of arlacel and of mineral oil; (*b*) the purified lipopolysaccharide somatic antigen of *Salmonella typhosa*; (*c*) commercial pertussis vaccine.

Mice vaccinated with the methanol extract in association with either one of these adjuvants still exhibited undiminished immunity when challenged with virulent tubercle bacilli many months after vaccination.

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March 13, 1956

[5] Dubos, R. J., and Schaedler, R. W.: J. Exper. Med., 1956 (in press).

Correspondence

A SIMPLE DEVICE FOR THE MICROCULTURE IN BLOOD OF TUBERCLE BACILLI IN PATHOLOGIC SPECIMENS

To the Editor of the American Review of Tuberculosis and Pulmonary Diseases:

In our laboratory we are using very satisfactorily, for the routine detection of tubercle bacilli in sputum or in other materials, the method of Pryce, i.e., the microculture in blood of smears made on glass slides.

Though we find the original Pryce method satisfactory, recently we have found it more convenient, for practical purposes, to sink the whole slide into a Petri dish containing an appropriate amount of blood. However, due to the increasing number of tests that we perform daily, the method results in too large a space being occupied in the thermostat by the Petri dishes, a problem which becomes particularly important when tests for susceptibility to antimicrobials are required, for which from ten to twelve dishes must be used for each sample.

To avoid this and other inconveniences related to the use of Petri dishes, we have made a very simple device (figure 1).

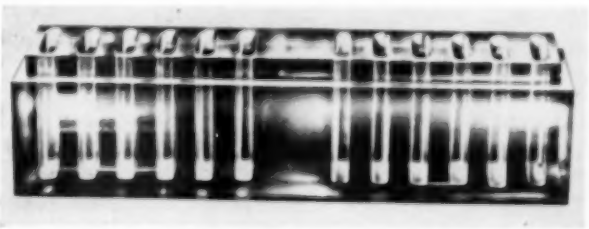


FIG. 1. The container for microculture in blood

The material is a transparent synthetic polymer. The size of the entire block is 25 by 5 by 9 cm. The holes are deep enough to contain a slide of 7.5 by 3 cm., the size most commonly used.

Another advantage is the small amount of blood which is necessary to fill up the single holes (10 ml.), compared with the 20 to 25 ml. which are needed for a Petri dish of an appropriate diameter. A cover of the same material as the block prevents evaporation of the blood during the seven to eight days of incubation at 37°C.

Each of these containers can be used for twelve slides from different patients for diagnostic purposes or, in turn, for one test for susceptibility to antimicrobials. For the latter purpose, the twelve holes are each filled with 10 ml. of blood. In the six holes on the left, appropriate amounts of streptomycin are added in order to obtain the concentration of the antimicrobial wanted in each hole. In the first five holes of the second series, ap-

propriate amounts of isonicotinic acid hydrazide are added. The last hole to the right is filled with blood to which no antimicrobial has been added (control slide).

Much time is saved by this device, both when compared to the original method of Pryce and to the use of the Petri dishes.

Cleaning and sterilization of the entire block after use is also very easy.

Sincerely yours,

Sanatorio L. Sacco-Vialba
Milano, Italy

Pietro Croce
Niveo Luridiana

January 16, 1956

Books

MEDICAL RESEARCH: A MIDCENTURY SURVEY. Vol I: American Medical Research in Principle and Practice; Vol. II: Unsolved Clinical Problems in Biological Perspective. Edited by Esther Everett Lape and Associates. Vol. I, 765 pp.; Vol. II, 740 pp. Published for the American Foundation, Little, Brown and Company, Boston, Massachusetts. Price, $15.00 for both volumes.

These two volumes present, in attractive format and lucid scientific detail, a broadly comprehensive picture of the principles underlying medical research, the methods by which it is practiced, the manner in which it is fostered, and the objectives it is designed to attain. It represents the fruition of fifteen years of intimate study, sponsored by the American Foundation and carried out by Esther Everett Lape and a small staff of professional associates, with the counsel of a committee of consultants representing some twenty medical fields.

Primary emphasis is laid on the essentiality of basic investigation for the advance of medical research. The utter dependence of applied investigation on fundamental knowledge gained in patient pure research in physics, chemistry, and biology is shown with compelling logic backed by a wealth of supporting detail. The account is of fascinating interest, altogether aside from its instructive value, bringing to the reader in a facile way, in spite of the technical complexity of its subject matter, informative pictures of modern research on metabolism and growth, cancer, chronic infectious illness, viral diseases, cardiovascular disorders, infertility, alcoholism, and schizophrenia. Tuberculosis is well covered. The account, rapid but discriminating, touches a wide range of tuberculosis research from fundamental investigation to applied chemotherapy. A penetrating section is devoted to the metabolic relations of host and parasite; indeed the necessity of basic metabolic research is emphasized for every disease covered.

A valuable feature of the monograph is its sweeping exposition of the sources of support for medical research. Recognition is extended to the leadership of the National Tuberculosis Association as a fund-raising body and operating agency in the promotion of education and research in its field. The volume should be read by progressive physicians, investigators, public health workers, administrators of organizations devoted to health and welfare, and all others concerned with the advance of medicine; they will find it richly rewarding.

ESMOND R. LONG
PEDLAR MILLS, VIRGINIA

ANTIBIOTICS ANNUAL 1954–1955. Edited by Henry Welch and Felix Marti-Ibanez. Pp. 1,154, Medical Encyclopedia, Inc., New York, 1955. Price, $10.00.

Antibiotics Annual 1954–1955 represents essentially the proceedings of the Second Annual Symposium on Antibiotics which was held in Washington, D. C., in October, 1954, and consists of a collection of one hundred and seventy papers that were presented.

The greater part of the book is devoted to the use of antibiotics in the therapy of infectious diseases of man. Numerous reports are presented on the clinical applications of the so-called broad-spectrum antibiotics, especially tetracycline. A number of papers on erythromycin, tuberculosis chemotherapy, the influence of corticoids on infection, oral and long-acting penicillin preparations, and drug reactions are also included. Those who

believe that bacitracin, chloramphenicol, and polymyxin B have not been fully exploited as clinically useful drugs will be encouraged by the renewed interest in these agents.

It is reassuring to find that, in the face of several accounts of the problems presented by microbial resistance, fourteen new antibiotics are reported. Similarly stimulating are the several reports of investigative activity in the field of fungal and neoplastic diseases. Nystatin has few significant side effects in man and is very active against experimental mycotic infections. It appears to be worthy of clinical evaluation in human cases of systemic mycoses.

The use of antibiotics in the control of infections in animals and plants, the growth potentiating activity of various antibiotics in cattle and swine, and the effect of antibiotics in increasing egg production and fertility in hens are the subjects of several articles and serve to indicate the wide range of usefulness of antibiotics in animal husbandry and botany.

This collection of papers is recommended for review by those persons with more than a general interest in antibiotics.

EDWARD W. HOOK
ATLANTA, GEORGIA

BACTERIOLOGY AND SEROLOGY. Selected Examination Methods for the Bacteriologic and Serologic Laboratory (in German). Second edition, completely revised. By L. Hallmann. Pp. 850, Georg Thieme Verlag, Stuttgart, Germany, 1955. Price DM 58.50.

Two separate sections of this book contain several chapters on general morphology and biology of bacteria, their localization in the human host, the methods by which they are obtained from body fluids and secretions, and the general principles of laboratory techniques, including sensitivity tests and bacteriologic examinations of water, milk, and other foods. In an appendix are discussed transportation of samples, dye substances for staining, various reagents, and first aid in cases of laboratory infections.

The principal and largest section of the book (approximately 440 pages) deals with the special examination methods and tests for all types of microorganism, viz., cocci, bacilli (including spore-formers), spirillae and treponemas, rickettsiae, viruses, fungi, and protozoa. About 130 pages are given over to a short presentation of basic immunologic concepts and descriptions of important immunologic laboratory tests of practical importance.

The chapter on mycobacteria begins with some remarks on acid-fastness. The different types of *Mycobacterium tuberculosis* are listed. Five methods of skin testing with Old Tuberculin Koch (scarification, intracutaneous and subcutaneous injection, puncture, and patch test) and the conjunctival tuberculin reaction are described. The morphologic and tinctorial properties of the mycobacterium (also Much's granula) are discussed in detail. In the discussion are included fluorescence microscopy and several methods of sputum concentration. Methods for the preparation of various liquid and semi-solid culture media are given, and the growth of the bacteria in these media is described. The diagnostic animal experiment is detailed in regard to the site of inoculation, the course of the disease, and the autopsy findings. Directions for the examination of urine, gastric contents, pus, exudates, tissue material, feces, blood, and milk are also given. The Dubos-Middlebrook reaction is dealt with in the serologic section of the book.

Similarly, highly adequate discussions are presented for all other organisms which might be of particular interest to the chest physician (fungi, viruses, et cetera), and to other medical practitioners, specialists, and laboratory workers in general. As a matter of

fact, the strength of the book lies in its wide scope; it will undoubtedly serve as a comprehensive reference text. Numerous tabulated surveys, schematic illustrations and graphs, together with many good and well-reproduced pictures, contribute a great deal to this aspect of the book. The print is clear and the paper is of good grade; the volume is of convenient size and format.

Unfortunately, there are some features of the book which reduce its usefulness. Because it tries to cover a wide field, it is sometimes necessarily defective. For example, there is no mention of the viruses of warts, condylomas, vaccinia, milkers' nodes, or of the rickettsiae of Colorado (spotted fever and rickettsial pox). A discussion of the life cycle of *Treponema pallidum* is also omitted.

In general, all factual directions for bacteriologic and serologic procedures are much more exact than the clinical or interpretative parts of the book. This is shown by instances such as the favorable opinion of the specificity of the Dubos-Middlebrook reaction or by the argument that the treponema immobilization test has little practical value because of its technical difficulty. Another disadvantage of the book, at least for non-German users, is its understandable yet nevertheless disturbing preference for German brands of chemicals, stains, and reagents which are, of course, often difficult to obtain outside of Germany.

In spite of faults of this kind, the book can be quite useful to bacteriologic and serologic laboratory workers in the performance of their daily routines, and it contains a great amount of up-to-date information for physicians with a sufficient knowledge of the German language.

KARL STEINER
NEW YORK, NEW YORK

BOOKS RECEIVED

CANCER OF THE LUNG. Pathology, Diagnosis, and Treatment. By Milton B. Rosenblatt and James R. Lisa. Pp. 330, Oxford University Press, New York, 1956, Price, $15.00.

TUBERCULOSIS IN THE ARMY OF THE UNITED STATES IN WORLD WAR II. An Epidemiological Study with an Evaluation of X-Ray Screening. By Esmond R. Long and Seymour Jablon. Pp. 88, Veterans Administration Medical Monograph, 1955.

SYNTHESIS AND PROPERTIES OF THE CHLORINATION PRODUCTS OF 4-AMINO-2-HYDROXY-BENZOIC ACID AND ITS METHYL ESTER. By C. Van Der Stelt. Pp. 63, D. B. Centen's Uitgeversmaatschappij N.V., Amsterdam, Holland, 1956.

OCKOVANI PROTI TUBERKULOSE (Vaccination Against Tuberculosis). By L. Sula. Pp. 282, Statni Zdravotnicke Nakladatelstvi, Prague, 1955.

ERGEBNISSE DER GESAMTEN TUBERKULOSE-FORSCHUNG. By St. Engel, L. Heilmeyer, J. Hein, and E. Uehlinger. Pp. 659, Georg Thieme Verlag, Stuttgart, 1956, Price, $22.85.

EUROPAISCHES SYMPOSION UBER DIE BEHANDLUNG DER SKELETT-TUBERKULOSE. By J. Kastert, S. Orell, and Ph. J. Erlacher. Pp. 115, Ferdinand Enke Verlag, Stuttgart, 1956, Price, DM 25.

RADIOIODIO E TIREOPATIE. By Domenico Scopinaro, Omero Ferrini, and Giancarlo Gambaro. Pp. 373, Archivo "E. Maragliano" di Patologia e Clinica, Genoa, 1955.

MEMORIA DEL PRIMER CONGRESO NACIONAL DE MEDICOS DE SOCIEDADES REGIONALES. Febrero 14 al 21, 1955, Havana, Cuba. Pp. 258, Cultural, S. A., Havana.

American Trudeau Society

Medical Section of the National Tuberculosis Association

Obituary

Unknowingly, two persons prepared obituary remarks about Arthur Walker. It was thought to be fitting and proper that both sets of remarks be published—The Editor.

ARTHUR MEEKER WALKER

1896–1955

In the pre-Christmas days of 1955, there were returned to Chicago the ashes of one whose family had survived and rebuilt upon the ashes of that Chicago. This background—of Chicago's "I will," and of memorable high station in the life of that cosmos and day[1]—was an over-riding fact in this man's search for perfection in whatever tasks he assigned himself. One-half of this background was reflected in his Edwardian courtesy, his grace, and erudition as conversationalist, correspondent, and editor. For the other half, he carried within him, to his unending torture, Alan Gregg's "air of expectancy" of great things to be done. But these great things he would not do except with utter integrity and scarcely penetrable anonymity.

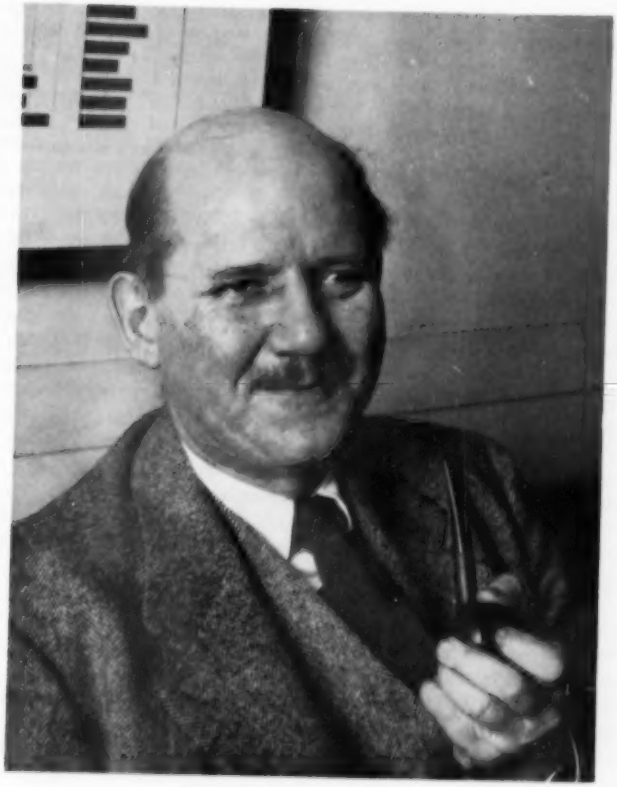
Arthur Meeker Walker was born on September 26, 1896, the son of James Ranson and Louise Meeker Walker of Chicago, at their summer home in Pittsfield, Massachusetts. His early schooling included the Chicago Latin School and Westminster School, Simsbury, Connecticut. He entered Williams College, Williamstown, Massachusetts, in the fall of 1915, joined Kappa Alpha, and left for the Reserve Officers Training Corps in the summer of 1917. By December he was Commanding Officer, Company C, 58th Infantry, 4th Division of the Regular Army. In August, 1918, he was wounded at St. Thibaut in the Chateau Thierry-Vesle campaign, but returned to the St. Mihiel-Argonne campaigns a month later, where he was wounded a second time, after which he was commissioned Captain and invalided to the United States.

He re-entered Williams College immediately upon discharge from the Ft. Sheridan Army Hospital and was graduated four months later with his class of 1919, completing in two and one-half years his four academic years. Yet he was editor of the Williams Record, Phi Beta Kappa, valedictorian of his class, and received his B.A. degree with the rare "maxima cum laude." Four years later, 1923, he was graduated at the top of his class and a member of Alpha Omega Alpha at Harvard Medical School. Such details of this early life are needed for an understanding of the meaning of the breathless "I will" of this singularly dedicated ego, and he counted them heavily in his own self-estimate.

His marriage to Sylvia Cabot occurred in Boston, November 29, 1924. Their four children were born in Philadelphia: Peter Walker, now of Westchester, Pennsylvania; Philip Cabot Walker, San Diego, California; Dr. James Elliott Cabot Walker, US Army Hospital, Osaka, Japan, and Maria Louise Walker MacIver, Glenview, Missouri. All three of the sons served fraudulently youthful terms abroad in World War II—only the British Ambulance Service would unbend its requirements for eyesight to accept one of these children. There are five grandchildren.

During his two-year internship (1923–1925) at the Massachusetts General Hospital, the staff began noting in his case histories the humorous parenthetical barbs that later made us all expectant readers of his VA-Army-Navy Quarterly Progress Reports. His

[1] Chicago, with Love, by Arthur Meeker, Little, Brown & Co., Boston, 1955.



ARTHUR MEEKER WALKER*

wide reading during this period attracted him to a program of investigative work in the field of renal physiology then in progress in the Laboratories of Pharmacology at the University of Pennsylvania. In the fall of 1925 he joined the "Chief," A. N. Richards, Professor of Pharmacology, and remained his disciple thereafter.

Richards, later, when President of the National Academy of Sciences, could write this of his work:

> He designed and conducted independently research which is generally regarded as unique in boldness of design and difficulty of execution. During his sixteen years in the laboratory, sometimes working alone and sometimes with others, he produced the data in five of the eleven categories by which this laboratory proved the validity of the "filtration-reabsorption" theory of urine formation in translucent kidneys of the amphibia—a proof which has met with general acceptance by physiologists throughout the world.[1] It remained for Walker to adapt the methods and develop the skills by

* Photograph provided through the kindness of William H. Feldman.

[1] RICHARDS, A. N., AND WALKER, A. M.: Urine Formation in the Amphibia Kidney, Harvey Lecture, January 17, 1935. Am. J. M. Sc., 1935, *190*, 727.

means of which comparable information was obtained concerning urine formation in the opaque mammalian kidney. In the face of incredible technical difficulties, he collected urine at various stages of its intrarenal elaboration by puncture of individual nephrons at various levels. In the analysis of the collected fluids and the identification of the exact sites of punctures, he was aided by his collaborators Bott, Oliver,[2] and McDowell; but the extraordinary feat—never since duplicated—of collecting uncontaminated samples in adequate amounts from single units of the mammalian kidney must be credited to Walker alone.

The collaboration in these experiments was his first venture in cooperative studies at long distance which characterized his later work with the chemotherapy of tuberculosis.[4]

His contributions to his country in World War II were characteristically prompt, vigorous, various, and of his own contriving. Before hostilities opened, he followed his "Chief" to Washington to become the assistant to the Chairman of the Committee on Medical Research of the Office of Scientific Research and Development. There he became familiar with material which after the war enabled him to write the chapters on "Penicillin," "Antimalarials," "Blood and Blood Substitutes," and "Insecticides and Rodenticides" in the book *Scientists Against Time*.[5] Except for one chapter by Detlev Bronk, these were the only contributions on medicine in this book which won for its editor, James Phinney Baxter, III, the Pulitzer Prize in History for 1947.

He capitalized on his World War I infantry experience by making himself "drillmaster" of the 20th General Hospital (University of Pennsylvania) when it was mobilized to Louisiana. In Burma with this hospital, he became bored with the duties of Registrar and transferred to Stilwell's Chinese infantry as Combat Liaison Officer. He concluded that he had proved only that he "could walk as far as the youngsters."

After the war, upon the conclusion of his duties as "Historian" of the Committee on Medical Research he was momentarily lost and accepted the position of Director of Chemotherapy, Tuberculosis Service, Veterans Administration, with great misgivings. At the time (February, 1946) he knew, or knew of, all the work and the workers in both penicillin and streptomycin. He confessed complete ignorance of, and some lack of interest in, tuberculosis, as well as a great mistrust of all phthisiologists. He concluded that his job was to help Chester Keefer and the Committee on Medical Research finish the evaluation of streptomycin in acute diseases and to cover the Veterans Administration's emergencies of miliary and meningeal tuberculosis. This last he did by setting up a storage refrigerator in his office and air-mailing emergency supplies to all parts of the country, using his own pocket money to pay the postage. On week-ends he would weigh, wrap, address, and walk with the supplies to the Washington postoffice to assure prompt dispatch. Thereafter, it was in the lap of the gods. Arthur had done what he could to save a life. But ignorance or mismanagement at the other end saddened and baffled him. Human failure he never understood and continued to refer to it as "wickedness," even in himself.

He was among the first to recognize that streptomycin must prove its worth in chronic tuberculosis or it would not be worth its cost to produce. It was in this scientific and somewhat capitalistic spirit that he (as anonymous secretary) organized in the spring of 1946 the first cooperative study of the effects of streptomycin on human tuberculosis.

[2] WALKER, A. M., AND OLIVER, J.: Methods for the collection of fluid from single glomeruli and tubules of the mammalian kidney, Am. J. Physiol., 1941, *134*, 562.

[4] WALKER, A. M., AND BARNWELL, J. B.: Clinical evaluation of chemotherapeutic drugs in tuberculosis, Ann. New York Acad. Sc., 1949, *52*, 742.

[5] Science Against Time, by James Phinney Baxter, III, Little, Brown & Co., Boston, 1946.

Thus, in June of that year began the first tests in what has since become the VA-Army-Navy cooperative study. His infantry experience in two world wars made intolerable to him all resolutions of repeated conferences to include the Air Force in the title and his quiet "I will" was allowed to over-rule the conference vote.

In early 1946, with so little streptomycin available and so little reliable information upon it, he thought both his "idleness" and his government pay excessive. Partially to remedy both conditions, he projected himself as anonymous "Editor" of the VA's Technical Bulletins Series 10. He objected to their title but no other niche could be found in the system of publications issued by this government agency. In the ten years of their monthly appearance he succeeded in keeping all other publications out of this series and in reducing the final sentence from: "By direction of the Administrator, Omar Bradley," to "Published and distributed by the authority of Roy Wolford, Chief, Professional Service." He also succeeded in eliminating all governmental editorial changes beyond his own—an achievement of unknown rarity to those out of government. Of these bulletins, the present Chief Medical Director, while still Dean of the University of Wisconsin Medical School, wrote: "The composition and organization of the materials comprising this Series set a high standard in American Medical Literature. The format is dignified and effective and the timeliness of the constituent articles is quite remarkable . . ." but ". . . I would not overlook the conspicuous editorship under Dr. Arthur Walker. His judgment and perspicacity are clearly reflected in the sustained quality of this outstanding Series."[6]

Dr. Middleton has, since the Editor's death, determined that the final bulletin showing his editorial skill shall be the last of that series and that any further such issues shall be given another name. As general as has been the appreciation of these bulletins, possibly the greatest tribute of all has been shown in letters from their distinguished authors expressing thanks for editorial fairness, judgment, consideration, and improvement of their original manuscripts. A final perverse triumph must be acknowledged. Of 112 bulletins published, this Editor, working in and for the Tuberculosis Service, allowed only one to be written by a phthisiologist and only three on the subject of tuberculosis—and one of these by a pharmacologist named Walker.

His legacy: On a recent intense evening, in talking to one of his "children"[7] he said these things about the cooperative study that he was so soon to bequeath to others. "We must prosecute this study in truth" so that all will "recognize the integrity that activates the leaders—following the dictates of their own hearts as well as their minds." We must acknowledge "the blush that comes into the eyes from known omissions." But we must further "look for the omissions the worker fails to see or to blush over. We must kill the intentional wickedness."

A great and troubled soul has passed among us and left its mark where he intended it—upon our goals. If we profit by this uncompromising life, his God may rest his soul in a peace it never found on earth.

J. B. B.

* * *

Arthur Walker died in his sleep on December 12, 1955. He was probably the best known individual among those interested in tuberculosis in the United States and he was certainly among the best beloved. To a truly extraordinary degree, Arthur Walker was a man of his times and participated in the fullest measure in the major activities of those times.

[6] LETTER: Dr. W. S. Middleton to American Medical Writers Association, January, 1955.
[7] Either his son Philip or Ross McLean.—Both were present September 16, 1955.

He was born in the closing weeks of the McKinley-Bryan campaign and grew up in New England and Illinois. He was at Chateau Thierry; he was at St. Mihiel and the Argonne, and was wounded on two occasions in that war. Yet, twenty-five years later he was with Stilwell in Burma.

Invalided home from World War I at the age of twenty-two, he completed his formal education at Williams College and was graduated as top man in his class at Harvard Medical College. After two years on the resident staff at the Massachusetts General Hospital, he went to the University of Pennsylvania where he worked under A. N. Richards, who was then conducting the brilliant series of investigations of the kidney which are now classic. Arthur Walker participated extensively in these researches and made an important contribution in adapting the technique for the study of the formation of urine so that it could be used in mammals as well as in amphibia.

When he returned from World War II, he spent four months as an historian for the Committee on Medical Research of the Office of Scientific Research and Development. In the course of this activity he contributed four chapters to the book *Scientists Against Time*, which received the Pulitzer Prize in History for 1947.

The last decade of his life was in many ways the one in which Arthur Walker exerted his greatest influence. He turned from working with a capillary micropipette in the laboratory to cooperative research on a nation-wide scale. Arthur Walker organized the United States Veteran's Administration cooperative investigation of tuberculosis chemotherapy. Starting with a group of physicians who for the most part were experienced clinicians but inexperienced in research, he labored hard to establish standards of criticism, evaluation, and expression. In very large measure, he shares in the achievements of this program which he served so well.

Like many a veteran warrior, Arthur Walker was a very gentle person. About him can most truly be said, as Justice Holmes said about his own generation and our Civil War:

> "In our youth our hearts were touched with fire. It was given us to learn at the outset that life is a profound and passionate thing."

W. McD.

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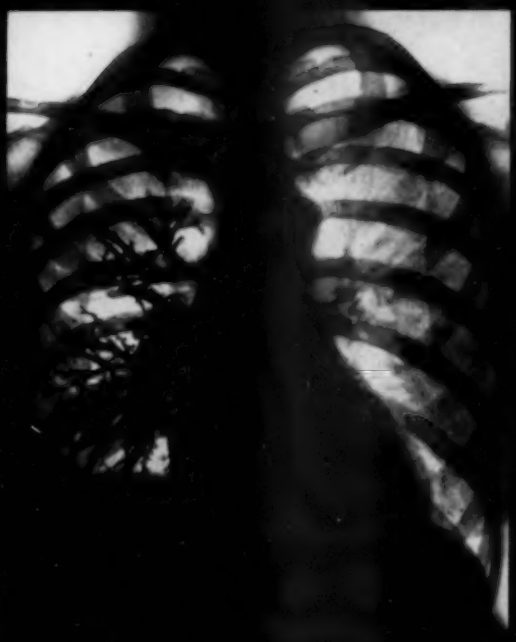

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1. Burrascano, J. J.: Sea View Hosp. Bull. *15*:149 (July) 1955.

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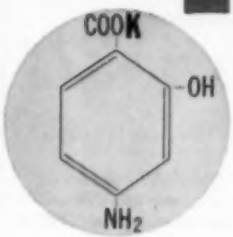

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1. *Molthan, L., Cohen, B. V., and Zarafonetis, C. J. D.: Clinical use of potassium para-aminosalicylate (KPAS). Am. Rev. of Tuberc. and Pulmon. Dis. 71:220 (Feb.) 1955.*

2. *Cohen, B. V., Molthan, L., and Zarafonetis, C. J. D.: Clinical Studies of Various Forms of PAS (with special reference to plasma concentrations). Read before the American Trudeau Society, Annual Meeting, Milwaukee, Wisconsin, May 23-27, 1955.*

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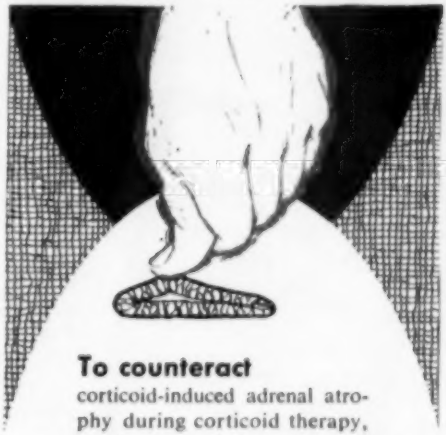

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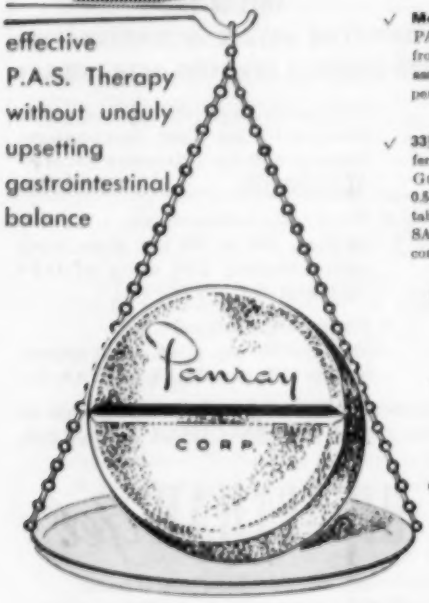

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